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Principles, policies and pipelines

The great Siberian pipeline row has caught out most Western governments (and the Reagan Administration in particular). The need is for a compromise. But intellectual work is also necessary.

The quarrel between the United States and Western Europe about the Siberian pipeline is another vivid illustration that nothing so confounds international relations as incomprehension, a failure to appreciate that other parties to a dispute may also have a case. But even when the origins of such disputes are insubstantial, the consequences may be as damaging as when the conflict is about some tangible issue, say a disputed piece of territory. Already, four European companies (three French, one British) have been put on an unprecedented blacklist to which United States corporations will be forbidden to supply goods. More European companies will no doubt soon join them, for there has so far been no break in the seeming determination of Western European governments that their domestic manufacturers should honour contracts for supplying equipment for the pipeline project. But in the long run, the financial damage to the companies concerned will be negligible compared with the damage done to relations between Europe and the United States. Paradoxically, even those in the Soviet Union who may at present be delighted at the apparent discomfiture of the Atlantic alliance may in the end themselves be discomfited. (Soviet readers, as important a part of any international journal's readership as any other, please note.)

Responsibility

President Ronald Reagan and his advisers must shoulder the prime responsibility for incomprehension. That Mr Reagan should have turned out to be a more zealous advocate than his predecessor of the use of economic sanctions to bring political pressure on the Soviet Union is not of course surprising, for it was well advertised in advance, but even Mr Jimmy Carter followed a similar course after the appearance of Soviet forces in Afghanistan at the beginning of 1980. The US government's present calculation is that economic sanctions against the Soviet Union, and in particular the denial of specialized supplies for the Siberian pipeline, will indirectly bring about a relaxation of the martial law regulations imposed in Poland last December. The questions whether the calculation is correct, whether the United States has reason to expect Western European governments to share its conclusions and objectives — and why President Reagan appears to have passed up the opportunity to find out at the June summit meeting at Versailles — are important but do not touch the central principle. It is the same with the questions that have been raised about the wisdom of the Western European governments agreeing to buy natural gas from the Soviet Union — strategic dependence in energy supply and the provision of a steady flow of hard currency over the decades ahead (see *Nature* 22 July, p.313). What matters, and what the United States appears to be incapable of understanding, is that these arrangements were concluded during Mr Carter's presidency, were then widely regarded in a very different light and could not now be abrogated retrospectively without causing serious legal difficulties for Western European corporations and even for their governments.

Mr Reagan's government is now busily making matters worse by insisting that one of the objectives of its present policy is to demonstrate to all concerned, Europeans as well as the Soviet Union, that the United States has reassumed the leadership of the Atlantic alliance. While, after several years of US indecision, that

message would ordinarily be welcome in Western Europe, its timing and its manner are inappropriate, not to say inept. For the governments in Western Europe that Mr Reagan now wishes to have follow the United States in being tough with the Soviet Union are, like his own, governments by the grace of their electors. And it has not been forgotten in Western Europe that the long and painful decline of Chancellor Helmut Schmidt's coalition government in West Germany dates from the time when he put his political future on the line in favour of President Carter's proposal to base neutron bombs in Europe — and then found that Mr Carter had climbed down without telling him in advance. Mr Reagan's present position on the pipeline, whether justifiable or not, is bound to seem to many as a reversal of previous policies without consultation, let alone agreement, and will thus strengthen the Gallic line towards the Atlantic alliance that has been rife in Europe for decades — the view that, over the long run, the interests of the United States are unlikely always to coincide with those of Western Europe, for which reason Western Europe had better plan for self-sufficiency.

European governments, whatever their political colour, will not be able to overlook the growth of this opinion, even if it should be misconstrued; to fail to do so might be to risk electoral defeat, even replacement by gallically-inclined successors. But even governments not now at risk, or which (like the British government, with the Falklands war behind it) keenly appreciate the importance of assistance from the United States, may find it impolitic to stomach the style of leadership now on offer. Unless the quarrel about the pipeline is quickly patched up, it is not entirely far-fetched that it could mark the beginning of the end of transatlantic arrangements for the defence of Western Europe. Strange though it may seem, that would not be the unmixed benefit for the Soviet Union that it would seem at first sight to be, for there is every chance that Western Europe living more by its own devices than at present would be a more awkward, if less predictable, customer than it is now.

Retreat

Retreating from the present position will demand more diplomatic skill than either the United States or Western Europe has shown in the past few months. Although the pipeline issue has been rumbling on since the beginning of the year, neither of the parties seems to have done what prudence would have required at the Versailles meeting — insisting that it should know for sure what was in the other's mind. Instead, the official communique includes a vague reference to the need for caution in dealing with the Soviet Union, given the troubling events in Poland in previous months. President Reagan seems diffidently not to have asked for a commitment to boycott the Siberian pipeline, and the governments of Western Europe to have been content to let sleeping dogs lie. Since then, embarrassment, indolence and more recently bad temper have been substituted for rational discussion. Until the imposition in mid-August of sanctions by the United States on French pipeline suppliers, it would have sufficed that the United States should have demanded of Western Europe an account of what it planned to do to implement the loose wording of the Versailles communique. Now, with opinions on both sides hardened, the only hope for a resolution of the quarrel is that there should be a serious attempt to hammer out a policy on



economic relations with the Soviet Union on which the governments concerned can agree, or agree to disagree. The more public that process, the more easily the United States could abandon as it must its sanctions against companies in Western Europe without losing face.

Agreement is too much to ask for, but a basis for civilized disagreement should be attainable. The underlying issue is what kind of relationship should obtain between the industrialized West and the industrialized East, partners in a more vexing dispute than over a mere pipeline. A few things are agreed, at least formally: there should be negotiations on strategic arms control, and neither side will sell defence equipment to the other. Two other issues are more fuzzy: the extent to which personal freedom is subordinate to or the basis for the integrity of the state, and the part that should be played in such a tense relationship by what would otherwise be considered normal commercial and cultural relationships. Broadly speaking, the governments of West and East differ radically on the first question (which is why they are at loggerheads), but the West is divided among itself on the second. It is possible to argue that the restraint of commerce will force illiberal regimes to become more liberal, but also that the opposite is the truth. The perplexity of British governments about the encouragement of sport in the Republic of South Africa is a proof that historical precedent is not a sure guide.

In such circumstances, the only acceptable basis for an understanding between the disputants of the West is that they should all know clearly where they stand on the restraint of commerce and of cultural relations and (knowing the hazards of ballot-box democracy) should be able to lay odds about where they may stand in future. On the face of things, there is much to be said for Andrei Sakharov's position (see page 199) that the questions of strategic arms control and of human rights (the nature of democracy) should be uncoupled and that the principle should also be extended to uncouple commercial relations on non-military commodities (such as natural gas) from the issue of personal liberty. For once restraint has gone so far that the provision of pipeline equipment is not allowed, the time would soon arrive when even the sale of ostentatiously unstrategic commodities (cereals, say) to the Soviet Union would be disallowed. Paper clips could just as easily be embargoed.

Ostensibly, none of this has much to do with the scientific community, but the contrary is the truth. The tale of how Sir Humphry Davy went on a scientific jaunt (with his new bride) through mainland Europe at the height of the Napoleonic Wars is almost too well known, while the fact that Pugwash could safely meet in Warsaw this August can easily be discounted on the grounds that it is no longer in a position to risk giving offence. Protests among Australian biochemists and other professional people that the federal government should not have put last month's meeting of biochemists at Perth in hazard by denying visas to two Soviet officials travelling as scientists are more to the point, if only because they illustrate how intimately government officials rather than professionals are now involved in the judgement of what should be done. (To its credit, the Australian National Academy of Sciences has done everything that might have been expected of it to protest.)

But such issues are too important to be left to government officials. Professional people in the strict sense should have more say. Natural scientists could (and therefore should) argue that their lives would be more interesting and therefore more productive if there were less, not more restraint. Economists (regarded as scientists proper in many places) have much to say about the fructifying all-round benefits of more trade, whatever its nature or with whom. Historians (also still considered to be professional scientists in most of mainland Europe) have important information to contribute about the unfolding of enduring events. If professional people are serious in their protestations that the present world is dangerous, they would be well-advised to turn their energies from saying that nuclear war would be cataclysmic to showing how to solve the more subtle but more urgent problem of East-West relations which has so perplexed the most powerful governments in the past few months.

Doing one's thing

What do the British Association, ICSU and the Falkland Islands have in common? Distraction.

Crisis is not everybody's agenda, and the British Association for the Advancement of Science duly met last week at the University of Liverpool for its annual jamboree. To nobody's surprise after 150 similar occasions, there were few surprises. Thus Dr Beverly Halstead rehearsed his previously advertised objections to cladistics, Dr W. Eldridge (Gould's collaborator on punctuated equilibrium, and now curator of fossils at the New York Museum of Natural History) replied and the London *Times* earned a familiar rebuke from Sir Andrew Huxley for having reported the affair as if it had sprung up anew. Most of those listening to the debate, like those attending the meeting as a whole, will have found the experience rewarding (for otherwise they would not have chosen to go). The British Association itself is in a more serious dilemma: it purports to carry the message from research laboratories to the world at large (and many daily newspapers still behave as if that were the case). For next year's meeting, the association is promising that it will have forged stronger relationships with learned societies in the United Kingdom, in the hope that its message will be more pointed. (There will still, of course, be anxieties about money.) Why does not this venerable institution stick to what it does well and have the courage to let others judge whether there are wider benefits and what they are?

The meeting of the International Council of Scientific Unions (KSU) at Cambridge (England) this week will have seemed to its participants to be in quite a different case. Should mainland China be admitted in preference to Taiwan (yes) and if so how (no terms specified in advance)? What should be done about the prospect that thoroughly international conferences will henceforth be impossible because of the assumption by governments that they should be consulted in advance? (The government of Australia last week gave the Australian delegation to the ICSU meeting terms of reference wide enough to be a basis for almost any negotiation at Cambridge, but added the proviso that in future is should have a hand in planning conferences of this kind). And Sir John Kendrew (once secretary-general of the union) was duly appointed president for the next three years. The international union is also short of money, partly at least because it is trying to accomplish goals for which it was not created.

The Falkland Islands are in much the same case. Earlier this week in London, Lord Shackleton (truly a relative of the polar explorer) produced for the British government (and the public gaze) his latest report on the economic prospects for these recently disputed islands. (The British government seems to have gone to war earlier in the year without having read the earlier version of the report, published in 1976.) Some of the recommendations are predictable — the only usable runway on the islands, for example, should be extended to 8,500 feet, thus allowing high-performance military aircraft as well as heavy civil transports to land safely. The proposed deep-water jetty is more of a surprise, as is the proposal that the ownership of land should be transferred, as is only just, to those who work it. In present circumstance, Lord Shackleton's proposal that there should indefinitely be a military garrison on the Falklands and that there should be a development agency modestly to encourage low-technology industries (fishing, knitting and the like) will similarly not cause consternation.

Predictably what remains unsaid is that the Falkland Islands, like the British Association for the Advancement of Science and the International Council of Scientific Unions, cannot be allowed to find their own level in the scheme of things (serving the membership or making arrangements that enable science to function internationally) because of external circumstances, the recent conflict, the shortage of money and the ubiquity of political considerations respectively. Is it not high time that such institutions (even islands) were allowed to get on with what they can do, and were not expected to do what is beyond them?

US fights Canada on Videotex

Moynihan plans retaliation for Ottawa law

Washington

An international war of nerves between the Canadian government and Senator Daniel Patrick Moynihan (Democrat, New York) may break into the open in September, affecting the fates of the Canadian, British and French videotext and teletext systems now competing for a prime position in the huge US market.

When the Senate's Finance Committee meets in late September, to take up a rather parochial bill dealing with US-Canadian broadcasting relations, it could well adopt a policy that would cripple Canada's videotext system, Telidon, as a contender in the US market — thus improving the chances of Britain's Prestel and France's Antiope, which until now had seemed dim.

The *agent provocateur* is Moynihan, who announced in June that he would try to introduce a clause into the bill (S.2051) that would prevent the treatment of any monies spent by US businesses to adopt Canada's Telidon system as a business expense. Many major US news organizations, such as Time-Life Inc., are well along the road towards adopting Telidon. Moynihan's clause has been denounced by an association of 100 US companies and individuals, the Videotext-Industry Association, as "an unprecedented and unreasonable kind of trade warfare".

Moynihan's clause is intended as a stick to beat the Canadian government and make it bend on a completely different issue. Moynihan's home state of New York includes several US broadcasting stations near the Canadian boarder that derive large audiences and considerable advertising revenue from Canada. The stations have been up in arms ever since 1976, when the Canadian parliament adopted a law, known as C58, that attempted to gain "cultural sovereignty" over what is broadcast in Canada. As Canada could not legally prevent incoming broadcasts (which are picked up by cable networks), it used the only legal means at its disposal and deprived Canadian companies of the right to treat as a business expense the cost of advertising on these US stations. The stations promptly called on Washington to do something. They estimate they have lost \$20 million annually in advertising.

Thus began what is now called the "border broadcaster's war". Both President Jimmy Carter and the office of the US Special Trade Representative have criticized the Canadian action. Congressmen from states along the border

have introduced legislation depriving US companies that advertise on Canadian broadcasting stations of the right to deduct their advertising revenues. But the Senate's bill (S.2051) is an insufficient weapon: the Canadian stations stand to lose only about \$3 million in advertising revenues, compared to the \$20 million lost to US stations under the Canadian law.

Thus Moynihan has hit on Telidon as an additional means of bringing Canada to the negotiating table. Canadian engineers worked closely with AT&T, NBC Inc, RCA Corporation and others to develop the "North American Standard" as a common standard to which all videotext and teletext systems should adhere. A key element in the system is the concept for

drawing graphics that is at the heart of Telidon. AT&T's own videotext system, which is not as fully developed as Telidon, would be compatible with this standard as would the French Antiope. These and other companies have jointly protested to Moynihan, citing the "severe negative consequences on the development of electronic publishing in the United States".

So far, the Canadian government had refused to be drawn on Moynihan's proposed action against Telidon but Canada will probably protest to the State Department if and when the clause seems likely to become US law.

The gigantic Times-Mirror newspaper empire, Time-Life Inc. has also protested strongly against the Moynihan proposal

Posts and statutes change at CNRS

A close henchman of Jean-Pierre Chevènement, the highly political French minister for research and industry, is to take up the top job in the management of basic science in France — the director-generalship of the Centre National de la Recherche Scientifique (CNRS). CNRS has a budget of FF6,000 million (£500 million) and a staff of 20,000, and most French scientists aspire to be supported by it. The appointment and major reforms in the statute of CNRS were announced last week in moves described by the minister as marking a "great change" in French science.

Some people, however, fear an increase in the political control of French science, something the CNRS directorate has long resisted on the principle that scientists are the best managers of science.

Certainly, there are some grounds for concern. Last year, the then president and director-general of CNRS resigned over the issue — when the minister tried to appoint his own nominee as director of social sciences. That post remains unfilled, as Chevènement appeared (remarkably) to have misconstrued the CNRS constitution.

But the constitution is now changed, so that CNRS scientific directors appointed only on the advice of the director general, rather than on his instruction and their powers will be increased in a general devolution of power in the CNRS management.

The new appointee to the CNRS director-generalship, 43-year-old physicist Professor Pierre Papon, denies, however, that the powers of his new post will be reduced. The increase in ministerial power "is only formal" he said on Monday. In practice, there will be constant discussion between CNRS and the ministry "as always". As for the great changes, CNRS will protect basic science, said Papon, but will now take more account of national cultural, economic and social needs.

Further, the powers of the director-

general will be increased in relation to the CNRS council, which will no longer make appointments and is renamed the council of administration. Claude Fresjacques remains president of CNRS and of this reduced council. One important power remains with the council, however: deciding the budget.

It is stressed at CNRS that there was no disagreement between Chevènement and the man Papon will succeed, Professor Jean-Jacques Payan. Although only ten months in his post, Payan has been enticed to a key job at the ministry of national education — where he will be responsible



Payan — universities. Papon — science.

for universities and research. Payan's move, in fact, may help to strengthen Chevènement's hold on university research (which, except through the research councils such as CNRS, is strictly outside his control) insofar as Payan himself is a one-time Chevènement nominee (for the CNRS director-generalship).

Other reforms in CNRS announced include the establishment of new, partially elected committees to advise the scientific directors and the creation of two new scientific directorates, one for the application of CNRS results, and one for publications and popularization. The constitution is also changed to allow profitable liaisons to be made between laboratories and industry. The changes are closely in line with the "law for research" voted by the National Assembly earlier this year (*Nature* 8 July, p.110).

Robert Walgate

fearing that the clause may be more destructive of US than of Canadian economic interests.

Moynihan's staff counters that the market for videotext and teletext is still so fluid in the United States that the big customers, who plan to use these systems to bring newspapers and news services into homes within a decade, can still choose the French or British system, that under development by AT&T or some other.

The US Federal Communications Commission (FCC), which has the legal power to affect the outcome by developing standards for this new industry or by endorsing some industry-proposed standard, is standing aside. In October 1981, FCC announced that the market should decide which systems were the best. The announcement, not yet a formal notice, heartened British Videotex and Teletex of New York, the company responsible for marketing Prestel in the United States. So long as FCC does not endorse a North American standard, Prestel still has a fighting chance. Senator Moynihan's clause would give it even a better one.

Deborah Shapley

Canada and yellow rain

UN expert group asked to act

Washington

This autumn will see a test of the United Nations' ability impartially to examine allegations of Soviet-inspired use of toxin weapons in South-East Asia. The Canadian government has submitted a report to the United Nations that gives credence to the charges and explains how they could be resolved. It will be up to the Group of Experts convened by the General Assembly to recommend to the Assembly whether or not to take up the report's suggestions. So far the Group of Experts has failed to reach any conclusion. The Soviet Union has denied that there is any toxin warfare in South-East Asia.

Canada has strongly supported the extension of the Group of Experts' mandate last year when its first investigation did not decide the matter. Refugees from Laos and Kampuchea say that helicopters and fixed-wing aircraft have been spraying poisonous gas that causes death to humans, animals and plants. If this is true, the Vietnamese, who

are presumably flying the planes and helicopters, would be in violation of the 1925 Geneva Protocol prohibiting the use of poisons in warfare. If, as is suspected, the Soviet Union is the source of the toxin weapons, it is in violation of the 1972 Convention on Biological and Toxin Weapons.

Independently of its active role on the issue in the United Nations, the Canadian government commissioned its own study of the matter. In February it asked Dr H. Bruno Schiefer, chief of the toxicology group in the Western College of Veterinary Medicine at the University of Saskatchewan, to try and resolve the veracity of the charges. Dr Schiefer made a two-week visit to Thailand — neither he nor the Group of Experts were allowed into Laos or Kampuchea to check reports first-hand or take samples. Like the Group of Experts, Schiefer interviewed refugees through an interpreter and took samples from border areas near the sites of the alleged attacks.

Schiefer concluded that the events reported to have taken place at the time of the alleged attacks "cannot be explained on the basis of naturally occurring phenomena". He corroborates an assessment made by the US Department of State earlier this year that toxin warfare was indeed being conducted there (see *Nature* 25 March, p291).

Schiefer first sought to resolve an apparent inconsistency. The poisons found in border areas, the particular tricothecene mycotoxins involved, act slowly and would have to occur in massive quantities to cause human death. Yet refugees seemed to say that clouds of "yellow gas" sprayed from the air caused death immediately. After close questioning of refugees, Schiefer concluded that the human deaths were not occurring immediately but that humans, animals and, plants in the vicinity were dying 10 to 14 days afterwards. This would be consistent with the level of poison reaching the ground in sprays.

Another problem has been that the tricothecene mycotoxins involved could not alone penetrate the human skin in such a fashion as to cause death. Schiefer began searching for possible agents that might be combined with the tricothecene to facilitate entry through the human skin. Because some of the refugees reported a garlic-like smell after the attacks, Schiefer suspects that dimethyl sulphoxide (DMSO) could be such an agent. The report suggests future field searches for DMSO and other possible agents.

The report also urges that a search should be made for the only known mycotoxin that could kill through ingestion, macrocyclic tricothecene, which thus would not need any additional agent to penetrate the skin.

Communication without compatibility

None of the six main videotex standards is fully compatible with any other. Each offers a different solution to the central problem of balancing quality of display against the cost of the terminal.

At the cheap end of the scale are the systems with "alpha-mosaic" graphics, producing pictures on the screen with a crude building-block appearance. The memory of alpha-mosaic terminals stores a limited repertoire of basic mosaic shapes, from which an incoming byte of data can extract a particular shape and place it in a particular slot on the screen. The result, although inelegant, is economic in the use of memory and transmission bandwidth. But the description of information in the database is terminal-dependent.

"Alpha-geometric" systems specify pictures in terms of basic geometric figures and coordinates of a point on the screen. Almost any shape can be created and placed anywhere on the screen, while picture descriptions are not generally terminal-dependent. But greater memory and processor requirements raise the cost of such terminals.

The leading videotex systems have the following characteristics:

- **Prestel:** An alpha-mosaic standard established in 1974 by the British Post Office (now British Telecom) in cooperation with the broadcasting authorities BBC and IBA. Internationally, there are more Prestel-type terminals than others. Extended graphics capability is being developed.

- **Antiope:** The alpha-mosaic standard developed by the French tele-

communications and broadcast authorities (DGT and TDF).

- **Telidon:** The first alpha-geometric standard (produced by the Canadian Department of Communications) has attracted much attention for its superior display capability.

- **32 × 16 US alpha-mosaic:** The central characteristic of this system — 16 lines of 32 characters — is based on the low-cost Motorola VDG microchip. It has been adopted by US cable television and microprocessor manufacturers.

- **CEPT:** An attempt by the Association of European Telecommunications Administrations to establish a unified European alpha-mosaic standard. The standard can interpret data according to either the Prestel or the Antiope schemes, depending on the terminal.

- **PLP:** AT&T's Presentation Level Protocol is not a standard in itself but is a vast package of options, from which AT&T will select a subset to be included in a terminal for mass production. The range of capability offered is so great that a comprehensive terminal would be of prohibitive cost. The importance of PLP is its flexibility and AT&T's formidable powers for production and marketing. Other suppliers are willing to modify their standards to be compatible with PLP. The CEPT standard is compatible with the PLP alpha-mosaic subset, but is restrained from change because of the many videotex and teletex sets already in use in Europe. Discussions with AT&T to modify its alpha-mosaic subset may prevent a permanent split between US and European standards. **Bronwen Maddox**

Schiefer also found that tricothecene mycotoxins do not occur naturally in the environment in the region — as they do in other places (such as Saskatchewan). Nor did he find evidence of the human illnesses that are associated with naturally-occurring tricothecene mycotoxins. His report recommends more systematic questioning of refugees so that a proper epidemiological survey can be made.

The mycotoxins in question are toxin weapons, whose production, development, stockpiling and transfer is forbidden by the 1972 Convention. Use of the weapons in warfare is prohibited by the 1925 Geneva Protocol. There has been some confusion about whether to count the

mycotoxins as chemical or biological weapons because although chemicals they are the products of living fungi.

Schiefer's report suggests that the agent involved may be not only the pure chemical but the chemical and fungus mashed together. It recommends that future field work should look for evidence of the fungi that produce the poisons.

The Canadian government has not formally endorsed Schiefer's report but has forwarded it to the Group of Experts. Its own study of the matter will continue, it says. The Group of Experts is due to report to the next General Assembly, which opens in mid-October and will stay in session about six weeks.

Deborah Shapley

Sakharov note makes waves

Pugwash survives Warsaw

Last month's Pugwash Conference in Warsaw — the thirty-second in the twenty-five year history of the Pugwash movement — stressed, once again, the special responsibility of scientists to help "devise means to limit and eventually reverse" the arms race, and their "major responsibility" for disseminating knowledge about the meaning and implication of their work. As is customary, the conference concentrated on major issues of arms proliferation and control, destabilizing factors such as the possible use of food and energy strategies for political means and current or recent world conflicts — Namibia, the Falklands, Iraq-Iran, Afghanistan and Lebanon. But what the council's concluding statement called "the deteriorating international climate" was reflected in two topics which did not appear on the official agenda — a letter to the Pugwash movement from Academician Andrei Sakharov and the meeting of council members with General Jaruzelski.

The Sakharov letter was not originally intended for the Warsaw meeting but for the Pugwash Silver Jubilee Conference in Canada earlier this year. It seems to have been delayed in transmission, however, and shortly before the Warsaw meeting came into the hands of one of the intending participants, Dr Joel Primack from the University of California.

Under Pugwash procedure, because Sakharov had not himself been invited to the conference, the letter could only be circulated as a background document. Not surprisingly, there was considerable Soviet opposition — one Russian proclaiming that since twenty million Soviet citizens had died during the Second World War, it was inappropriate for "anti-Soviet propaganda" to be distributed at a Pugwash meeting. Finally, however, normal procedure prevailed.

The Sakharov letter in fact contained little new. It censured the negative stance taken by Soviet delegations at international meetings where "in all discussions of critical problems, they always behave as

well-disciplined functionaries of one gigantic bureaucratic machine".

As in several previous statements, Sakharov gave "absolute priority" to the achievement of international security and disarmament. In spite of his known opinion that there should be no absolute linkage between disarmament and human rights, he also appealed to the participants to speak out in defence of prisoners of conscience in the Soviet Union.

While generally sympathetic, the conference could not agree with Sakharov's view that "the West would be unable to withstand the forces of the USSR and its camp if nuclear and thermonuclear weapons were excluded from the balance". Rather, the conference concluded, the concept that the Warsaw Pact conventional forces are significantly more powerful than those of NATO needs "searching reexamination".

The problem of linkage seems to have underlain many of the doubts expressed by participants about the meeting of the Pugwash Council with General Jaruzelski but meetings with the head of state or government of the host country are a Pugwash tradition.

Pugwash participants say they put a number of searching questions to the general on the implications of martial law, the future of Solidarity and the fate of internees, and that he predictably replied that the present "state of war" was necessary to defend the state and economy from anarchy, but that "a number of democratically orientated social, political and economic mechanisms" had nevertheless arisen.

Even so, some participants felt that the very fact of the meeting might in some sense be interpreted as conferring a kind of approval on the current military regime. Jaruzelski, himself, however, carefully avoided any such claim, saying only that the choice of Warsaw for the Pugwash meeting was an expression of respect for the Polish nation, and of trust in its wisdom".

Vera Rich

European community research

Spending money

Brussels

The brief Belgian summer has not distracted officials at the European Commission from putting together plans for the new European science strategy agreed at the ministerial meeting on 30 June. Three papers have been presented to the Council of Ministers, EEC's decision-making body, covering the reform of the research and development programme of the joint research centres, the preliminary phase of the Esprit programme on information technologies and the first phase, for 1983, of the long-term plan to stimulate the scientific and technical potential of the European Community.

The last of these projects, however tentative, is the most interesting. The Commission is proposing that the Community should spend \$5 million to stimulate research in seven selected fields, thus paving the way for spending on a larger scale in the period 1984–87. For next year, the plan is that funds should be spent on a handful of fashionable fields of enquiry — new applications of cell and molecular biology, composite materials, mathematical analysis applied to optical problems, the behaviour of materials in the presence of combustion, non-destructive testing, interface phenomena and transitory effects in climatology. Information technology is already catered for by the Esprit programme.

If the Commission's proposals are accepted by the Council of Ministers, the research ministers at their meeting arranged for 30 November will invite applications for research funds in respect of both the pilot programme for research and development and for Esprit.

One of the attractive features of the pilot programme is that funds will be available for multi-national projects, including seminars and workshops, as well as for the travel and research expenses of scientists working at foreign laboratories.

The research ministers are unlikely to be as pleased with the Commission's paper on the joint research centres, the latest revision of the rolling three-year programme. The rising cost of the Super-Sara project on reactor safety is the perennial stumbling-block, especially for those governments that were dubious about the project at the beginning. The Commission now argues that work on this project should be pushed ahead so that the past investment in it will at least yield some information in time for it to be used in the design of light-water reactors, but it is not yet clear whether these arguments will prevail. For the rest, however, the Commission has proposed that the work of the four joint centres should be brought more into line with the new framework proposed for the general support of research and development. **Jasper Becker**

Ariane in space

What next?

Last week's failure of the fifth Ariane flight is bound to have repercussions on the launcher's commercial prospects. But officials at the European Space Agency (ESA) are still hopeful that the fault that caused the failure in the rocket's third stage will prove to be no more than a manufacturing error, thus reassuring customers of Ariane's basic soundness. Three previous successful flights are taken as testimony that the fault probably does not lie with the design.

ESA is keen to point out that the rocket's first and second stages worked perfectly. But the third stage, which was carrying two satellites into geostationary transfer orbit, suddenly lost power after about 4 seconds and brought the satellites back down to Earth. A preliminary analysis of telemetry data recorded at a station in north-east Brazil has revealed a sudden failure in a gear controlling the flow of cryogenic propellant from the liquid oxygen and liquid hydrogen pumps. Nobody yet knows, however, precisely what went wrong with the gear.

Whether the failure was due to a simple error or to a more ominous design fault, sorting it out is bound to delay the future Ariane launch programme. A lengthy delay could postpone the launch of Exosat, ESA's X-ray astronomy observatory, from its scheduled date of next November to June or July next year. Exosat can only be launched during specific launch windows around December and midsummer.

Although a delay to Exosat could upset Europe's hard-done-by X-ray astronomers, ESA is bound to be more worried about the effects of delay and uncertainty over Ariane's reliability on the launcher's potential commercial customers. Intelsat, the international owner of the Intelsat series of telecommunications satellites, for

example, is due to launch three of the Intelsat 5 series on Ariane next year. Although the organization has made little response yet to last week's failure it will be watching the next Ariane launch keenly before making any firm commitments.

Meanwhile, ESA is having to decide how to make amends for Marecs B and Sirio 2, the two satellites lost last week. Sirio 2, a telecommunications satellite of primarily Italian design was uninsured, but the insurance money from Marecs B could be used to assemble quickly Marecs C, possibly for launch at the end of 1983 or the beginning of 1984. Inmarsat, the International Maritime Satellite Organization which had planned to lease channels on Marecs B for maritime communications in the Pacific, says that it can continue its present service in the area with the US Marisat satellite. But it is likely to take up ESA's offer to assemble and launch as quickly as possible the Marecs C replacement.

Amendments to the launch programme could delay the transition of the Ariane programme from ESA's control to that of Arianespace arranged before last week for July or August next year. Arianespace seems likely to take longer than expected before earning money. The company's prospects also seem to depend more than it must have hoped on the success or otherwise of the space shuttle, due to make its fifth (and first operational) flight in November.

Judy Redfearn

Spina bifida

Trials ahead

The Medical Research Council (MRC) is soon to start trials to investigate the role of folic acid and other multivitamin supplements in the prevention of neural tube defects (NTD). The programme is controversial because of its ethical implications. Women invited to participate will already have conceived one spina bifida baby and are therefore at risk of conceiving another. In order to understand the effects of dietary supplements, women in the control group will not be given multivitamin and folate supplements.

The government is to give £300,000 for the first three years of the trials. Although many scientists and doctors believe that multivitamin supplementation is important in the reduction of NTD, MRC says that new trials are necessary because previous experiments have not provided strong enough evidence for such a link. Experiments on folate and multivitamin supplementation at the Universities of Cardiff and Leeds are considered not to have been properly randomized. The women who participated were mainly from the middle classes and therefore at lower risk anyway.

The MRC programme will involve 3,000 women at 20 centres. There will also be centres in Israel and Australia. The women

will be offered full antenatal screening facilities and the centres will have to be approved by local ethical councils. The volunteers, who are planning a pregnancy (pills are taken before and after conception), will be randomized and divided into four groups. The first will receive pills containing minerals plus multivitamins, the second minerals, multivitamins and folate, the third minerals and folate and the control group minerals only. This design will permit a comparison between the multivitamin effect and the folic acid effect.

The National Childbirth Trust has written to the Minister of Health, Mr Kenneth Clarke, expressing its worries about the trials. It says that there is already sufficient evidence to link folic acid deficiency with spina bifida and point out that the women participants will have a one in four chance of being in the control group and therefore at risk. In Britain one in 20 women who have already conceived an NTD baby conceive another.

Ethical worries about the trial's implications hang on how well the women will be briefed. The Association of Spina Bifida and Hydrocephalus (ASBAH) agrees that there is a need for more scientific evidence and that this cannot be done without further trials. Its support is qualified, however, by the proviso that the participants must be adequately briefed. At present MRC decides what information is given to women volunteers.

Dr Nicholas Wald of the Radcliffe Infirmary in Oxford, who is to coordinate the trials, stresses that the women will be invited to participate. But he points out that even if they were not fully briefed the trials would not necessarily be unethical. He argues that the trial is ethical primarily because there is a broad balance between the likely good of the treatment and the possible harm.

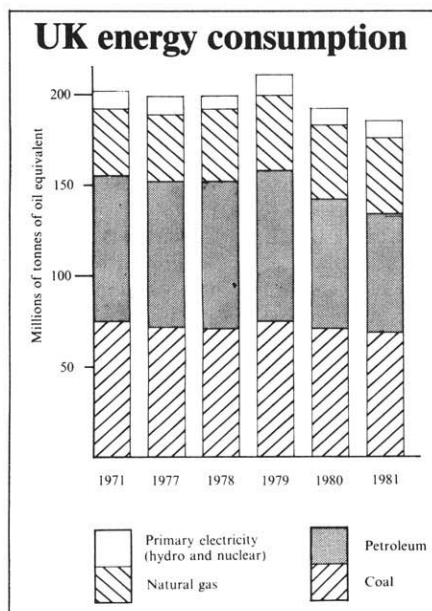
No one is willing to be explicit about what volunteers will be told. While women in the control group will not receive multivitamin or folate supplements, it should however be pointed out that such supplements would not necessarily be recommended by a general practitioner or by ASBAH in any case. ASBAH hopes that the MRC trials will establish what dietary advice should be given to women at risk who plan to become pregnant again.

Jane Wynne

Helsinki agreement

Monitoring stops

The formal disbanding, last week, of the Moscow "Helsinki Monitoring Group" is little more than an acknowledgement of a *fait accompli*. Since its foundation, six years ago, to monitor Soviet observance of the Helsinki Accords, the group has been subject to ever-increasing official pressure. In 1978, its founder and original chairman, Dr Yurii Orlov the physicist, was sentenced



to seven years in a labour camp, plus five years Siberian exile, for his part in the group's activities. Since Dr Orlov's conviction the active membership of the group has been whittled down by successive arrests to, effectively, two persons, Elena Bonner, the wife of Academician Andrei Sakharov, and Sofia Kallistratova, an elderly lawyer. It was apparently an official warning to Mrs Kallistratova last week that she might soon be arrested and charged with anti-Soviet activities that led to the disbanding.

The Moscow Helsinki group has not been the only victim of repression. Other Helsinki groups throughout the Soviet Union, and in particular the Ukrainian group founded by Mykola Rudenko the science fiction writer, have been similarly obliterated by the imprisonment of their members. So has the Moscow "Working Group for the Investigation of the Misuse of Psychiatry for Political Purposes". The founder members of SMOT, the "Inter-professional Free Trade Union", have all been either taken into custody or expelled from the Soviet Union. Since the arrest of Dr Viktor Brailovskii on the opening day of the Madrid "Helsinki Review" conference in November 1980, the Moscow Sunday Seminar for refusenik scientists has been able to meet only on very rare occasions, and regular participants have been subjected to harassment. Brailovskii himself is now eligible for early release from his exile in Beineu (Kazakhstan), but has so far been unable to obtain the necessary character reference from the local authorities, which, it is understood, want specific authorization from Moscow.

Some forms of grass-roots dissent do seem to have survived, however. Mrs Albina Yakoreva, a computer specialist and founder member of SMOT, who was put on a plane to Vienna last month, reported that the free trade union movement has decentralized, but that at least twenty-one grass-roots branches are active, their main object now being the building up of a climate of opinion that will accept such fundamental democratic concepts as worker self-management. In Byelorussia, which was relatively passive during the upsurge of dissent of the early 1970s, underground initiatives protesting against the russification of Byelorussian culture have been reported. The three Baltic republics, Lithuania, Latvia and Estonia, which have developed their own traditions of dissent (the *Chronicle of the Lithuanian Catholic Church* has long been one of the most prestigious underground Soviet journals, while Estonia has staged protest marches and token strikes) have, in the past year, begun to coordinate their efforts on fundamental issues. Their most significant effort so far is a letter to the governments of the Nordic and Baltic countries expressing their support for the proposed Nordic nuclear-free zone, but urging that the ban be extended to the relevant areas of the Soviet Union.

Vera Rich

Nuclear waste reprocessing

Germans divide

Heidelberg

The reprocessing of nuclear waste has predictably become an issue in the Hesse and Bavaria elections, now in full swing. The Social Democrats, the major partners in the Bonn coalition with the Free Democrats but fighting separately in the elections for the *Länder* governments, shoulder the chief responsibility for having declared that each *Land* includes a candidate site. The Free Democrats, party to these decisions but united electorally with the more powerful Christian Democrats, are more or less immune from the attacks with which the "Greens" (the environmentalist party) threaten to enliven the elections.

The 14 nuclear power plants in West Germany now produce some 350 tonnes of spent fuel-rods a year. By 1990, 25 power plants will be producing 750 tonnes a year. Under contracts with the French state-controlled Cogema, the rods will be reprocessed at the Cap de la Hague plant in Normandy until 1985, after which the outlook is unclear. Pending the completion of a second 800 tonne per year unit at Cap de la Hague, West Germany will in any case have to take back the highly reactive waste

and put it somewhere.

At present, Cap de la Hague and Sellafield (as Windscale is now called) are the only two commercial reprocessing plants functioning, but there is a chance that the smaller plant at Mol in Belgium may reopen soon (see *Nature* 26 August, p.783). Worldwide, however, there is a desperate shortage of reprocessing facilities, with 200 nuclear power reactors operating and 150 under construction. Only the US interdiction of the reprocessing of spent fuel bridges the gap.

In West Germany, after the abandonment last year of the plan for a 1,400-tonne reprocessing plant at Gorleben in Lower Saxony for political reasons and after massive environmentalist protest, the industry-run organization responsible for reprocessing is considering three 350-tonne per year sites at Frankenberg (Hesse), Schwandorf (Bavaria) and Kaisersesch (Rheinland-Pfalz).

The final storage of wastes, however, remains the responsibility of the federal government. Geologists estimate that salt deposits underlie about a quarter of Northern Europe and in spite of protests and a recent controversial study which revealed unexpected depths of erosion of the salt dome during the last ice age, investigations continue.

Sarah Tooze

Biotechnology briefs

The Cetus Corporation announced last week the first lay-offs in its history and a substantial refocusing of its research and development on projects that would be likely to pay off in the short term. Forty employees have been laid off, and projects dealing with the production of high-purity fructose and energy and chemical processing have been stopped. Cetus raised a record \$107 million with its public offering of shares in March 1981, but Standard Oil of California, a major shareholder, has declined to continue supporting fructose work at the company.

Another biotechnology company with ambitions in the artificial sweetener field is about to make a public stock offering. The Genex Corporation of Rockville, Maryland, is expected to offer 2.5 million shares in late September or early October, which could raise as much as \$33 million according to documents that Genex has filed with the US Securities and Exchange Commission (SEC). Genex already has a production plant for L-aspartic acid, which is used in the production of aspartame, a sweetener marketed by the Searle Corporation that was recently approved by the US Food and Drug Administration. Genex is also interested in genetically-engineered production of calf renin, bovine, porcine and ovine animal growth hormones and various industrial chemicals. The company is 45 per cent owned by the Kopper Co. Inc. of

Pittsburgh, a major chemical concern. The company, which is one of the largest genetic engineering companies in the United States, has grown steadily through contract research, the income from which rose from zero in 1977 to \$3.9 million in 1981.

Eli Lilly and Co. of Indianapolis is expected to announce later in the month the availability of the first genetically-engineered product for human use, human insulin. The work has been carried out by Genentech Corporation of South San Francisco.

Meanwhile, Armos Corporation, also of South San Francisco, a company founded in June 1980 by Brian T. Sheehan, formerly of Genentech, has filed for bankruptcy in the San Francisco courts. Sheehan says that this will enable the company to reorganize and that several investors are interested in its potential.

Another Canadian entry into the biotechnology field is Alleelix of Mississauga, Ontario, whose scientific director, Derek C. Burke, is leaving the University of Warwick in the United Kingdom to work full-time for the company. Alleelix is a joint venture between the Canada Development Corporation, John Labatt Limited and the province of Ontario. Burke says that the assurance of Can\$100 million over the next ten years will prevent the company from having to "panic" in the quest for short-term genetically-engineered products.

Deborah Shapley

CORRESPONDENCE

No to quangos

SIR — Your leading article in *Nature* of 5 August ("James Watt and biotechnology", p.501) asks some important questions, which you imply that only the government can answer. The difference between James Watt's doings and ours is that he was free from committees and we are not. I agree that to go back to James Watt would do no harm, provided that in going back we dismantle the bureaucratic structure that strangles our communications. Mr G.H. Fairtlough may understand the links between the Medical Research Council and the British Technology Group (and the National Enterprise Board and National Research and Development Corporation) and Celltech, but few others do. Things would work so much better without these complicated bureaucracies and we and Mr Fairtlough would be free to compete or collaborate with each other, and free to collaborate with the research scientists.

You ask how the government can give British industry a sporting chance of survival. The answer (towards which the present government is working) is to leave it alone. Take the committees and the quangos (and the formal channel of communication between the Department of Industry and the University Grants Committee) off our backs.

S.J. STARKIE
(Managing Director)

Xoma (UK) Ltd,
London SW17, UK

Cold war

SIR — I am afraid that E.G. Dimond's suggestion to exchange ¼ million young people between the United States and the Soviet Union as an alternative to arms race (*Nature* 15 July, p.220) would be more unacceptable to the Soviet Union than a tenfold increase in arms buildup by the United States. Why? It would be labelled as a "philistine ploy (1) for mass infiltration of the Soviet Union by CIA agents and (2) for infecting Soviet youth with decadent bourgeois ideology to use them later as a Trojan horse to disrupt the fabric of the socialist society". This is the essence of the tragedy why every effort to reduce tensions, promote détente and so on must inevitably end in deadlock.

VIT KLEMES

National Hydrology Research Institute,
Ottawa,
Ontario, Canada

China syndrome

SIR — In *Nature* of 19 August (p.746) you published a paper entitled "Age of the Lufeng, China, hominoid locality". One of the authors, Qi Guo-qin, appears on the contents page as Q. Guo-qin, which wrongly assumes Guo-qin to be the surname, and is a common mistake among Westerners.

The convention in Chinese names places the surname first, followed by the given names, which are usually hyphenated. The author is therefore Mr Qi not Mr Guo-qin. The Chinese tend to use their names in full, and the shortened form is made by dropping the given names for well known people, as in "Chairman Mao"!

There is, however, a tendency among Chinese exposed to the West to adopt our convention and place the initials of their given names before their family name. Perhaps they have become fed up with our getting their names mixed!

As scientists, the Chinese name convention should not strike us as odd — it is, after all, the same as the Linnean nomenclature!

J. A. IRVING

Dulwich, London, UK

Scrapie a "gene"?

SIR — In *News and Views*¹ R.H. Kimberlin commented on the recent suggestion by Stanley B. Prusiner of the University of California, San Francisco, that the scrapie agent is a novel proteinaceous infectious particle².

The search for the scrapie virus proposed by Cuillé and Chelle in 1938³, has been continuous. An early trickle of papers became a torrent when Chandler transmitted the disease to mice in 1961⁴. There have been so many variations on a virus theme that the virus hypothesis now lacks absolutely nothing, except a virus. One response to this impasse has been to call the virus "unconventional"⁵; an ingenious solution, for is not a square an unconventional circle?

In pursuing our suggestion in 1967 that the scrapie agent might be a small protein⁶, my colleagues and I applied fractionation procedures to scrapie tissues. Every experiment included control inocula of normal tissue treated precisely as the scrapie tissue, and diagnosis of scrapie was rigidly based on the presence of characteristic histological lesions in the brain⁷. These technical points are emphasized for two reasons. First, because few other investigators of the disease have used normal tissue controls in more than the occasional experiment, and very few have examined histologically the brain of every animal in every experiment, whether or not it showed clinical evidence of scrapie; this latter procedure is important in detecting scrapie agent in high dilution, or in unexpected circumstances. Second, because use of this experimental routine may explain why we apparently detected scrapie agent in tissues of some non-scrapie animals.

Our findings were published in *Nature* in 1968⁸. In brief, in over 150 experiments scrapie did not occur in any of 2,640 mice injected with unfractionated tissue from normal sheep, goats or mice (an observation matching that of many other investigators). By contrast, scrapie occurred occasionally, and unpredictably, in mice injected with fractionated tissue from non-scrapie animals. The possibility existed, therefore, that fractionation had released a scrapie-producing factor from non-scrapie tissue.

The obvious interpretation of our findings was that normal tissue had been contaminated with scrapie. To examine this criticism, we carried out further experiments in which attempts were made purposely to contaminate normal tissue inocula. By using unwashed glassware etc. we certainly produced some contamination, but the distribution of scrapie was different from that observed after injection of fractionated normal tissue⁹.

If, for a moment, it be accepted that we did

indeed detect scrapie agent in normal tissue, an explanation can be offered for the apparent enigma of replication without nucleic acid that faced us initially, and now faces Prusiner. The agent may not, in fact, replicate as the disease progresses; instead — as we suggested in 1968 — replication may be simulated by an unmasking process of a particle already present. Again, if scrapie agent were a component of normal tissue, there would be a simple explanation for the widely observed but obscure phenomenon that the scrapie agent does not apparently stimulate antibody. If the agent were "self" no antibody would be expected.

In recent years, research on scrapie in sheep has largely been bypassed in favour of the experimentally-produced disease in laboratory animals, for the obvious reason that few laboratories are equipped to handle large numbers of sheep. In consequence, it is not widely recollected that many outbreaks of naturally-occurring scrapie in sheep have been associated with close mating to improve agriculturally desirable breed characteristics¹⁰. In 1974 I reported the apparently spontaneous occurrence of scrapie in sheep selectively bred for high susceptibility to experimental scrapie¹¹. The significance of genetic make-up in susceptibility to scrapie in sheep is beyond dispute (for example ref. 12).

It may not be far-fetched, therefore, to suggest that the scrapie agent is an inhibited particle in normal tissue, released genetically in the naturally-occurring disease in sheep.

I.H. PATTISON

Newbury, Berks., UK

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To the point

SIR — Having read Cedric A.B. Smith's letter proposing a new system for representing numbers (*Nature* 12 August, p.600), I am afraid that it cannot go unchallenged. He not only suggests a totally confusing system of numbers and letters, but goes on to contradict himself.

He states "0.700 means exactly the same as .7", but is wrong since .7 is correct to one decimal place and 0.700 is correct to three places. And surely ".7" can be easily confused with "7" — referring back to an earlier paragraph in his letter "... what makes it worse is that especially in handwriting (and sometimes also in print) not only may dots and commas be difficult to distinguish reliably but also they may be so faint as to escape notice altogether".

MOHAMED S. NANJJI

Department of Biochemistry,
National Heart Hospital,
London W1, UK

NEWS AND VIEWS

The unacceptable face of Minoan Crete?

from Keith Branigan

THE Minoans were a civilized people; by soon after 2000 BC they were living in spacious houses with bathrooms, toilets and drains, and they decorated their living rooms with colourful frescoes depicting the birds, animals and fish which inhabited their beautiful island and the seas around it. Only the legend of the Minotaur, half man, half bull, which annually devoured twelve Athenian youths sent as tribute to King Minos, has cast a shadow on this idyllic scene — until the summer of 1979 when two dramatic discoveries were made in excavations only a few miles apart in central Crete.

At Knossos, the home of the Minotaur, the butchered remains of two young lads were found in a basement room of a Minoan house. Careful study of the bones suggested that cannibalism provided the only satisfactory explanation of all the evidence. Meanwhile, at Arkhanes, less than 10 km to the south, below the fallen walls of a Minoan temple destroyed by earthquake at about 1700 BC, the skeleton of a boy aged about 18 years was found sprawled across an altar, a bronze knife lying on the body. Again, a careful examination of all the evidence led the excavators to the inescapable conclusion that here was a Minoan human sacrifice. These two remarkable discoveries have naturally attracted a great deal of attention and comment, and alternative explanations to cannibalism and sacrifice have been offered; but do the alternatives fit the observed facts?

Details of the Arkhanes temple were published by S. Elliott in 1980 (*The Athenian*, 22 March 1980), with extensive quotes from an interview with the excavators, I. and E. Sakellarakis. Altogether four skeletons were found in the temple: one, a man, in the ante-chamber, and three (including the sacrificial victim) in the western of the three cult chambers. The other two skeletons in this room were those of a woman, lying face down and legs splayed, and of a man, fallen on his back and with his arms raised to his chest. These three skeletons are thought to be the remains of people (priests?) struck down in the temple at the time of its destruction by a major earthquake, which is well evidenced elsewhere in central Crete around 1700 BC. The fourth victim is thought to have died in

a different manner for four reasons.

First, unlike the others, he lay not on the floor but on a stone platform or altar. Second, he was not spreadeagled like the others but lay on his side with his legs tucked tightly up against the body — an unusual position suggestive perhaps of his being trussed up, but certainly not the position of someone caught by falling masonry or timber while trying to escape the building. Third, the ceremonial bronze dagger lying across his body is suggestive of sacrificial activity. Finally, the bones on the lower side of the body were stained a much darker colour than those on the upper side, and it has been suggested that this preserved traces of the manner in which the man died — from a severed artery on his left side, through which the blood drained, first from his upper right side, and eventually from his left side.

The collective evidence is impressive but not incontrovertible. In particular, pathologists in this country are unconvinced by the differential discoloration of the bones, which cannot be ascribed to the position of any supposed wound. The sacrificial weapon, too, presents some difficulties because it is not a dagger but a spearhead, and although it could be used to kill someone, it would be a cumbersome implement to use for sacrifice, particularly in the manner suggested — piercing the carotid artery of a person lying on their side on a raised altar. It might also be argued that the body fell, rather than lay, on the altar or platform where it was found. Only the position of the body is difficult to reconcile with this person being a fourth victim of the earthquake rather than of a priest's sacrificial knife, and until we have definitive reports with photographs of that position, we cannot be certain how critical to the interpretation the position of the skeleton really is. At present, the case for human sacrifice at Arkhanes has to remain 'unproven'.

The evidence from Knossos is well documented in articles and photographs published by the excavator, Peter Warren [in *Sanctuaries and Cults in the Aegean Bronze Age* (eds Hagg, R. & Marinatos,

N.) 155, 1981; *Archaeological Reports for 1980–81*, 73, 1981]. A small basement room contained a deposit dating from about 1500–1450 BC, amongst which were 300 fragments of human bone. On analysis, these proved to be the remains of two boys, aged 8 and about 11 years respectively. Although examination of the skulls and bones suggested both children were in good health at the time of death, it also revealed 27 bones on which there were clear knife marks, several of them well away from the ends of bones. The bones were subjected to microscopic examination and it was concluded that the marks had been made by repeated cutting or sawing (possibly with a sharp obsidian blade rather than a bronze knife) and there was no evidence for either chopping the bones or for scraping the bones longitudinally.

The discovery of these gruesome remains in the basement of a house raises three obvious possibilities. The first is that the bodies result from an undiscovered crime. This proposition can be repudiated by observing the nature and position of the cut marks. None results from chopping or slashing, none results from blows struck with a heavy implement. Apart from marks on the torso, there are also cut marks on the legs and arms, which are difficult to explain in terms of murder wounds. Most significant, however, are the multiple cut marks at a single point — one clavicle, for example, has five marks criss-crossing each other at one point, and another three 1 cm away; these must represent repeated and careful cutting or sawing rather than eight very precisely aimed blows on the single collar bone. A second explanation is that we have in this basement the remains of two secondary burials — that is, the bones removed from an initial burial place for burial elsewhere or perhaps for use in ritual. Secondary burial was certainly practised in Crete, but there are no other examples of children being subject to this treatment, and such evidence as we have for the practise is found, not surprisingly, in Minoan cemeteries, not in town houses. In any case, how does secondary burial explain the cut marks?

Warren plumps, somewhat unwillingly, for the third possibility — that we have the first evidence of Minoan cannibalism. He argues persuasively that the only explanation consistent with the nature and

Keith Branigan is in the Department of Prehistory and Archaeology, University of Sheffield, Sheffield S10 2TN.

position of the cut marks is that flesh was being cut from the bodies, and although in this case it cannot be demonstrated, the likelihood is that the flesh would be eaten. There are some further clues which support the proposal. For example, among the childrens' bones there were also some sheep bones, including a third cervical vertebra bearing cut marks consistent with the sheep having had its throat cut. In a room across the corridor from the childrens' bones, a storage jar was found containing burnt earth, snails and more childrens' bones, including one with cut marks. Are these too food remains? The ritual nature of the deposit in this second room is undoubted, with a remarkable collection of miniature vases of various shapes, each with a small hole perforating their base — typical libation vessels. Certainly, if we have evidence for cannibalism, we should look for a ritual setting (see, for example, Harris, M. *Cannibals and Kings*, 1978), whatever the real reason for the eating of human flesh may have been.

There are, however, some unusual features of the discovery which ritual cannibalism cannot explain. The first is the incompleteness of the skeletons; neither skeleton was anywhere near entire and, in particular, in neither case was there evidence for the lower legs. If the bodies had flesh removed for eating, obviously the bodies must have been newly dead; the absence of substantial parts of the skeletons cannot be explained by advanced decomposition and disarticulation. The bodies must have been slaughtered and butchered elsewhere, with only parts of the bodies being brought here; or, the butchering was done here and the missing parts removed to other places. Such events are possible, but they raise many new questions if we accept them. Second, some of the bones bearing cut marks come from parts of the body unlikely to have been eaten. Thus, all three shoulder blades, one of the two collar bones and two of the jaw bones show cut marks. If we recall that the evidence of the marks clearly points to cutting or sawing rather than chopping, and that we are therefore thinking not of butchery but of removing meat from the bone, then it is difficult to believe that in these instances we are looking at evidence for cannibalism; this is particularly true of the jaw bones.

The most economical explanation of all the observed facts that can at present be offered is that we have here evidence for post-funerary ritual involving human bones; that is, that the bones had been removed from a primary burial area and were being used in, or prepared for use in, a further ritual. The removal of bones from funerary contexts for further ritual use is attested elsewhere in prehistory (Piggott, S. *The West Kennet Long Barrow*) and there are hints of this activity in Crete by the third millennium BC (Blackman *Kretika Chronika*, 199, 1973). In particular,

Blackman refers to bones found at the Ayia Kyriaki tholos tomb (*Annual of the British School at Athens*, 77, in the press) which were clearly and cleanly cut through at both ends. The cutting or sawing marks on the bones from Knossos could be traces of similar activity, although none of the bones

found in the basement has been completely cut through.

On the evidence from Knossos, we cannot assert that the Minoans did not practise human sacrifice or cannibalism, but the onus of proof must still lie with those who believe that they did. □

Of cannibals and kin

from J.S. Jones

THERE has grown up in biology the comforting supposition that nature is not really red in tooth and claw; that animals behave, in general, in an altruistic or at least a polite fashion to other members of their own species, and that animals belonging to the same species rarely do serious damage to each other. Just how far this is from the truth is revealed in a recent account of cannibalism in natural populations¹.

Cannibalism has been recorded in more than 1,300 species, and is often the primary cause of mortality. Ninety per cent of fish fry may be eaten by conspecifics, three-quarters of crow eggs and nestlings cannibalized by adults, and a quarter of all lion cubs killed and eaten by older animals. In viviparous salamanders, a few young develop teeth which they use, while still unborn, to devour their less well-endowed sibs, and adult hyaenas often eat the corpses of others which they have killed in territorial battles. The female of most species is more cannibalistic than the male. The flesh of one's own species may be a major source of food; even in man — where anthropophagy has been recorded in some sixty cultures — human flesh was sometimes the second most important item of dietary protein. Some animals are particularly enthusiastic cannibals. Walleye fish, *Stizostedion*, eat each other 'tail first', and chains of up to four fish engaged in simultaneous cannibalism have been seen².

How can this apparently unadaptive behaviour evolve? The answer is no doubt complex and involves a variety of selective forces which differ from case to case. The individual benefits are obvious enough, for a cannibal not only obtains food but also reduces the competition. In ground squirrels, *Spermophilus beldingi*, for example, infanticide by unmated females leads to previously occupied nest burrows becoming available³. There may also be population benefits as cannibalism enables populations of desert scorpions and other animals to persist when food is short in a fluctuating environment⁴. However, the most interesting aspect of cannibalism is the insight it may give into evolution by kin selection.

It is often claimed that the behaviour of J.S. Jones is in the Department of Genetics and Biometry, University College London.

animals towards other members of their own species is influenced by their degree of genetic relatedness. Altruistic behaviour, in which one individual decreases his own fitness in order to increase that of others, is believed to be particularly likely to occur when donor and recipient have a high proportion of their genes in common. Unfortunately, it is usually difficult to identify a particular behaviour as altruistic, and impossible to assess the costs and benefits to the individuals involved in the interaction. Cannibalism epitomizes selfishness. It is hence the converse of altruism, and many prove a better test of the theory of kin selection, as it is easier to identify cannibalism and to measure the nutritional or other benefit gained by the attacker.

In some circumstances, patterns of genetic relatedness do seem to influence the evolution of cannibalism. Adult eggs of the flour beetle, *Tribolium confusum*, are often eaten by larvae and pupae by adults. Crosses between populations with different cannibalistic tendencies show that the behaviour has a genetic basis⁵. Experimental populations founded by members of the same large and variable base population attain very different population size under identical conditions — largely because of differences in the rate of pupal cannibalism⁶. In high-productivity populations, 85 per cent of pupae survive to become adults, while in populations with low productivity and equilibrium size the survival rate is only 5 per cent.

Wade⁷ has recently made use of this genetic variation in behaviour to show that the evolution of cannibalism is affected by degree of relatedness as predicted by kinship theory. Laboratory populations of *T. confusum* larvae were offered eggs from full sibs, from half sibs or from unrelated individuals. Larval females which ate eggs laid on the average one egg more per day on reaching adulthood than did non-cannibals. After eight generations, the egg cannibalism rate was lowest in the full-sib group, intermediate in the half-sib and highest in the populations in which the larvae had been offered eggs from unrelated beetles.

Kin selection may also be involved in controlling cannibalism in vertebrates. In

the lemming *Dicrostonyx groenlandicus*, males kill the offspring of unrelated males, but do not attack their own young⁸. Young males of the gerbil *Meriones unguiculatus* will normally kill and eat any pups. However, experimental exposure to a pregnant female inhibits this behaviour⁹. As gerbils are monogamous and territorial, any pups which a male meets after an encounter with a pregnant female in nature are likely to be his own and hence to share a high proportion of his genes. In mice, however, in which males will mate with any available female, destruction of young by adult males continues throughout life. Pregnant female mice exposed to a novel male reabsorb the fetuses which have resulted from a previous mating; this 'prenatal cannibalism' may have evolved in response to the high probability of the young being eaten at birth by the unrelated male¹⁰. Such kinship effects may influence the demography of vertebrate populations. In pike, attacks on unrelated individuals mean that fewer young are produced in ponds founded by mixing two full-sib families than when members of only one family are used¹¹.

Kin selection is particularly likely to reduce cannibalism in organisms whose local populations are relatively inbred, so that their members have a rather high proportion of genes in common. Wade's experiments on *Tribolium* show this clearly, as an avoidance of sibling cannibalism evolved only in inbred populations. In experiments in which there was random mating among the evolving stocks, no differences appeared among populations offered related or unrelated eggs. Computer simulations show that even a small amount of inbreeding greatly hastens the evolution of altruistic behaviour¹². It is often assumed that the social groups found in large mammals represent locally inbred populations and that their close kinship leads to the evolution of altruism and the avoidance of cannibalism among their members. However, detailed observation of individual mating patterns or the analysis of relatedness using genetic markers in lions and primates show that the members of a social group are not always particularly closely related to each other^{13,14} so that their behaviour cannot be explained simply by assuming that kin selection is operating.

A less ambiguous method of testing the importance of kinship effects has recently

become available with the realization that many hermaphrodite animals reproduce by self-fertilization¹⁵. This represents very close inbreeding. It might therefore provide an unusually direct way of examining the role of kinship in aggression and cannibalism, as in those species in which both selfing and outcrossing forms occur in the same population, outbreeders should be more likely to attack their relatively distant relations than are selfers

to destroy their close kin.

It is already clear that to cannibalize unrelated animals may increase the probability of survival of an aggressor's own genes. This may be an important factor in the evolution of behaviour, however much it conflicts with the widespread view that the struggle for existence is usually tempered by avoiding fatal interactions between members of the same species. □

Increasing atmospheric carbon dioxide and its consequences

from John G. Lockwood

NUMEROUS forecasts of the increase in atmospheric CO₂ that will result from the burning of fossil fuels have been made. The IIASA Energy Systems Programme¹ predicts an atmospheric CO₂ concentration of 430 to 500 p.p.m.v. in 2030 while The World Climate Programme² suggests values of 410 to 490 p.p.m.v. in 2025. Concentrations as low as 200 p.p.m.v. have been estimated for the end of the last glacialiation^{3,4}, even though levels may have been considerably higher in the more remote geological past. Recent observations show that the global mean CO₂ concentration in the atmosphere has increased from about 315 p.p.m.v. in 1958 to about 335 p.p.m.v. in 1979, a considerable increase over nineteenth century pre-industrial levels estimated at about 290 p.p.m.v.

Armed with forecasts of future fossil fuel needs and CO₂ production, how do we estimate the effect that these increasing concentrations will have? At a recent gathering in Norwich*, Gates (Oregon State University) argued that the problem is best approached using general circulation models (GCMs), which have the ability to portray many of the nonlinear feedback processes which serve to regulate atmospheric (and hence climatic) changes. The first application of a GCM to the CO₂ climate problem⁵ used an idealized geography with a swamp-like ocean and annually averaged insolation, and predicted that a doubling of atmospheric CO₂ would result in a 2–3K increase of globally averaged surface air temperature. This first attempt also showed the warming to be several times larger in high latitudes than at the Equator, and the globally averaged precipitation to be increased by a few per cent.

In subsequent simulations with GCMs

including more realistic geography and seasonal variations, generally similar results (global temperature increases of about 2K for a doubling of CO₂) have been found; such GCMs indicate that the largest warming will occur in the winter in high latitudes, with the summer precipitation shifted slightly polewards along with increased surface dryness in mid-latitudes. Flohn (University of Bonn) noted that high-latitude warming could lead to the disappearance of the perennial Arctic sea-ice and changes in the mass balance of the Antarctic ice sheet. In contrast, the zero-order, purely optical effect of doubling atmospheric CO₂, according to Kandel (CNRS), would be an increase of about 0.2K in surface temperature, but depending on what is assumed about various feedback mechanisms, such as atmospheric temperature, humidity, lapse rate, evaporation and clouds, this zero-order sensitivity may be multiplied by a factor ranging from 0.1 to 50.

Detection studies are now being directed by Wigley and Kelly (University of East Anglia) towards isolating those parts of observed climate fluctuations that are attributable to increasing atmospheric CO₂. This involves detecting what is at present a relatively weak signal in the presence of considerable noise. Climatic changes during the past century can partly be explained by increasing atmospheric CO₂ with contributions from volcanic activity. However, on the basis of the results so far it seems unlikely that a CO₂ signal will be detected with high confidence before 1990. Nevertheless, it looks as though CO₂-induced warming could become a major climatic factor by the middle of the next century. □

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*The Royal Meteorological Society summer meeting on 'Climatic effects of increasing atmospheric CO₂' was held on 15–16 July 1982.

John G. Lockwood is in the School of Geography, University of Leeds, Leeds LS2 9JT.

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A growing role for reverse transcription

from Harold E. Varmus

THE viruses resembling human hepatitis B virus that were recently discovered in other animals — woodchucks¹, ground squirrels² and domestic ducks³ — have begun to yield their anticipated harvest. In the June issue of *Cell*, J. Summers and W. Mason (Institute for Cancer Research, Philadelphia) make a strong case, based upon work with the duck virus, for the novel idea that RNA-directed DNA synthesis ('reverse transcription') is central to the life cycle of hepatitis B viruses⁴. The claim that RNA tumour viruses (retroviruses) are not the only animal viruses to use a reverse transcriptase may have implications that reach well beyond virology, since a growing constellation of eukaryotic genetic elements — various pseudogenes^{5,6} and repetitive sequences⁷ — appear to depend upon reverse transcriptases of unknown provenance for their existence or amplification. It is not necessary, however, to venture beyond the human hepatitis B virus itself to appreciate the significance of the new findings: this virus is currently replicating in about 200,000,000 people⁸ and infection is strongly associated with the most common

reactions *in vitro*. In infected duck livers, on the other hand, Mason, Summers and their colleagues recently observed that single-stranded putative intermediates in DNA synthesis were composed exclusively of 'minus' strands¹³. The asymmetrical properties of the two strands of hepatitis B viruses appear inconsistent with semi-conservative patterns of DNA replication; instead, the picture more closely mimics retroviral DNA synthesis, in which a virion-associated enzyme synthesizes a 'minus' DNA strand from an RNA template and a 'plus' strand from the 'minus' strand¹⁴.

Past efforts to understand the life cycle of hepatitis B viruses and make sense of such asymmetries have been frustrated in part by the failure of any member of this virus class to grow in cultured cells. Summers and Mason⁴ have circumvented this problem using materials from ducks naturally infected with the duck hepatitis B virus (DHBV). Their experiments required two crucial reagents isolated from cytoplasmic extracts of infected livers: viral cores that exhibit 'endogenous' DNA polymerizing activities, and deproteinized viral nucleic acids that work as templates in reactions 'reconstructed' with a retroviral reverse transcriptase. Synthesis of DHBV 'minus' strand DNA, in either the 'endogenous' or the 'reconstructed' reaction is resistant to the DNA intercalating agent, actinomycin D, whereas synthesis of the 'plus' strands in either reaction is sensitive to the drug. Furthermore, the growing 'minus' strands are partially associated with RNA and largely sensitive to a single-strand-specific nuclease, whereas the 'plus' strands are present entirely in DNA-DNA duplexes. These findings have been interpreted to mean that 'minus' strands are synthesized from templates of DHBV RNA and that 'plus' strands are synthesized from templates of 'minus' strand DNA.

The general picture that emerges from this work reveals the life cycles of hepatitis B and retroviruses to be temporally permuted versions of each other. Infectious particles of retroviruses contain viral RNA; viral DNA is synthesized in the cytoplasm early after infection and used in turn as a template for repeated synthesis of genomic RNA later in the infection. Infectious hepatitis B virus particles, in contrast, contain incompletely double-stranded DNA, the product of reverse transcription; viral RNA must be made during an early step in the life cycle and used as template for reverse transcription within cytoplasmic cores in the late phase.

The gross similarities in replication strategy, however, should not obscure a host of intriguing questions that address contrasting aspects of DNA synthesis, viral persistence and oncogenesis by these two

classes of viruses. Unlike retroviral reverse transcriptases, the hepatitis B DNA polymerases have not yet been solubilized to allow the enzymological study they deserve, so these proteins and their activities remain basically unknown. (The enzymes, incidentally, are probably virus-coded, since both the human¹⁵ and woodchuck¹⁶ hepatitis B genomes maintain a large unassigned open reading frame adequate for a protein of molecular weight about 90,000.) One clue to the enzymatic differences may reside in the mechanism of priming. Retroviruses use host tRNA to prime synthesis of the first strand of DNA; hepatitis B viruses, on the other hand, have an unidentified protein linked to the 5' end of 'minus' strand DNA¹⁷. Is it possible that the primer is a protein, as in the synthesis of adenovirus DNA¹⁸?

Both retroviruses and hepatitis B viruses establish persistent infections, but again important differences are evident. Retroviruses perpetuate their genomes in infected cells by efficient and precise integration of DNA that resembles a transposable element and forms a suitable template for long-term synthesis of viral

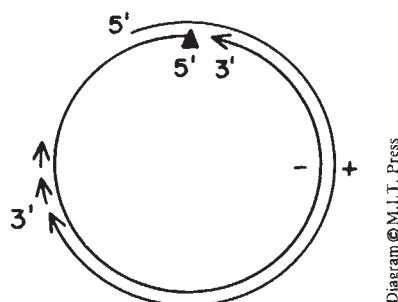


Fig. 1 Genome structure of hepatitis B-like viruses. Δ , covalently bound protein.

fatal cancer of man, primary hepatic carcinoma⁹.

Among the clues that led Summers and Mason to consider a replicative mechanism involving reverse transcription were the asymmetrical features of hepatitis B virus DNA, identified in both virus particles and infected cells. The viral DNA genome, as found in particles isolated from sera, is composed of one 3.2 kilobase strand of fixed chemical polarity (the 'minus' strand) base-paired with a shorter 'plus' strand of variable length¹⁰. (Because the 5' ends of these two strands invariably overlap by about 300 bases¹¹, the genome is in a circular configuration, although neither strand is itself a closed circle; see Fig. 1). A DNA polymerizing activity in virus particles, first discovered in the human virus several years ago by Kaplan *et al.*¹², extends only the 'plus' strand during

Harold E. Varmus is a Professor in the Department of Microbiology and Immunology, School of Medicine, University of California, San Francisco, California 94143.

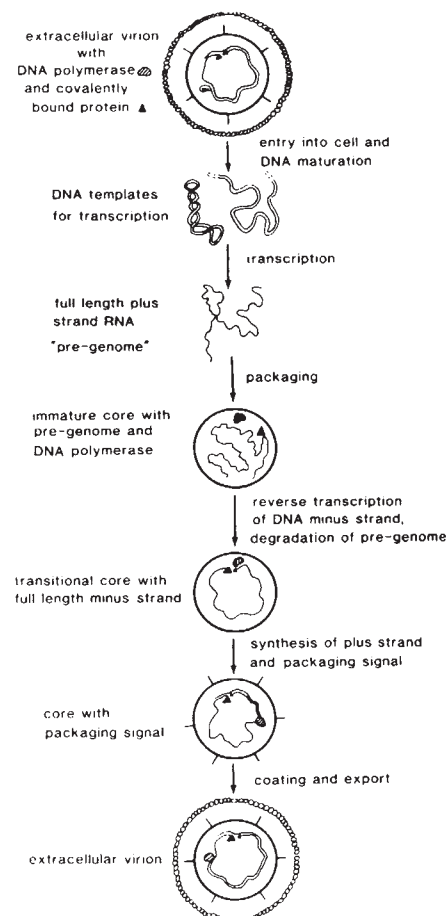


Fig. 2 The pathway for replication of the genome of hepatitis B-like viruses proposed by Summers and Mason⁴.

RNA¹⁴. Little is known about the integration of hepatitis B DNA during productive infection of the liver; but the first detailed account of integrated viral DNA in woodchuck liver tumours, again by Summers and his colleagues¹⁹ in the same issue of *Cell*, reveals complex rearrangements within multimeric viral inserts. Similar rearrangements have been found in integrated DNA from a human hepatoma cell line (W. Rutter, personal communication). This picture is not conducive to a belief that integrated DNA

has a role in the life cycle. But if ordered integration does not occur, how is the genome maintained during persistent infection? In particular, what is the template for synthesis of valid copies of viral RNA?

A final comparison of retroviruses and their new cousins is at once the most obvious and the most mysterious: are the hepatitis B viruses truly oncogenic agents and do they use mechanisms recently discovered to influence the development of tumours by retroviruses? □

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serotonin, noradrenaline, thyrotropin-releasing hormone and somatostatin — are preserved, indicating that death of nerve cells, rather than a change in afferent inputs, initiates the disease.

Attempts to produce animal models to mimic the pathology, the movement disorder, or the neurochemistry of the disease have received wide attention in recent years. Despite the large number of neurologic mutations that have been described in rodents, particularly mice, none has been identified which shows the key pattern of normal development, followed by cell death in a selected region of the central nervous system. Such mutants, if discovered, might provide a useful clue to the factors that lead to maintenance of cell integrity and an opportunity to study local trophic influences or lack thereof.

The local injection of neurotoxins, the most important of which is kainic acid^{2,3}, has provided a way of mimicking some of the effects of Huntington's disease. There is evidence that the principal neurotransmitter in the corticostriatal pathway, which forms a major input to the striatum, is glutamate, an excitatory neurotransmitter. Kainic acid, a rigid, cyclic analogue of glutamate, has potent excitatory effects, and when injected locally into brain tissues that contain glutamate receptors, causes neuronal death. Cells die, but incoming terminals survive, and many of the biochemical changes of Huntington's disease are seen. Because the effects of kainate require an intact corticostriatal system (the drug is less effective in animals that have previously had surgical corticectomy), it has been proposed that an endogenous kainate-like substance may overstimulate the corticostriatal system with cell death arising from an excitotoxic effect. Kainic acid receptors have been described in the brain and Coyle and his associates³ are currently searching for an endogenous substance with kainate receptor binding capacity. Additional evidence for the importance of afferent inputs to the striatum in determining the neurotoxic effects of kainic acid is provided in the paper by Berger *et al.* in this issue of *Nature* (p.254). Depletion of serotonin by injection of 5,7-dihydroxytryptamine, a serotonergic neurotoxin, into the brain stem is shown to make striatal cells more resistant to kainic acid. The kainic acid model, although unlikely to provide a clue to the basic genetic defect in Huntington's disease, does hold some promise for assessing the effects of striatal lesions and perhaps for testing the effectiveness of various therapies.

An alternative approach has been to search for evidence of a generalized defect in cellular functions in Huntington's disease by investigation of peripheral non-neuronal cells more accessible to study. Initial indications of *in vitro* abnormalities in fibroblast growth rates and sus-

Huntington's disease: genetically programmed cell death in the human central nervous system

from Joseph B. Martin

HUNTINGTON'S DISEASE, characterized by involuntary movements and progressive dementia, is transmitted as an autosomal, dominant trait whose effect is not seen until the third or fourth decade of life. The tragedy of the disorder lies not only in the effects of the disease itself, but in the absence of any genetic marker to diagnose those at risk; hence transmission of the disease to offspring commonly occurs before symptoms develop. Any inquiry into the cause of the disease must tackle this central issue of how a gene mutation can permit normal development, integration and function of neuronal systems for some thirty to forty years and yet then bring about the relentless, regionally selective, premature death of neurones.

The caudate nucleus and putamen (striatum) are the most severely affected, but other regions of the basal ganglia, hypothalamus and brain stem are also involved. The loss of neurones in the striatum has not been seen to be accompanied by any cytopathology or tissue reaction and cells appear to die without inducing a local

response. Other cells and systems of the body appear unaffected. Dementia becomes severe, but its characteristics are strikingly different from those of the more common Alzheimer's disease, and at autopsy, although the cerebral cortex is diminished in overall size, no certain decrease in total numbers of cells has been unequivocally demonstrated.

How can one account for such a course of events and how can research strategies approach a solution to the problem? The discovery that the symptoms of Parkinson's disease are due to a dopaminergic deficiency secondary to degeneration of the pigmented nigro-striatal system provided strong grounds for investigating biochemical changes in Huntington's disease. Within the striatum and often in the neural projections from it, there is a decrease in the concentrations of the neurotransmitters, acetylcholine and GABA, and of the neuropeptides, substance P, enkephalin and cholecystokinin¹. These decreases in transmitter concentrations appear to be associated with the cell death since their concentrations remain normal in other regions of the brain. Tissue concentrations of other transmitters in the afferent pathways to the striatum — including dopamine,

Joseph B. Martin is Bullard Professor of Neurology in the Massachusetts General Hospital, Harvard Medical School, Boston 02114.

ceptibility to irradiation have not been adequately confirmed⁵. Studies of differences in red cell membranes by electron spin resonance and of abnormalities in $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ have not been replicated^{6,7}. To date one cannot point to any described abnormality of other cells which provides an indication or marker of the genetic defects.

Perhaps the answer will ultimately come from the new techniques of molecular genetics. The Huntington's disease gene is considered to have a penetrance of 100 per cent and the mutation rate is exceedingly low; indeed, no proven cases of sporadic mutation have ever been described which satisfy the criteria of proven parentage, with parents surviving to an old age without symptoms, pathologically proven Huntington's disease in the new case, and subsequent transmission to a child. The low mutation rate makes it possible and practicable to search for a DNA polymorphism which might be useful as a genetic linkage marker⁸. So far the

chromosomal localization of the defect is unknown but restriction endonuclease digests of DNA collected from the peripheral white blood cells of patients with the disease, combined with somatic cell hybridization, are being used to localize the polymorphisms to a specific chromosome. In the long term, it should be possible to survey each chromosome in the human genome sequentially, first for a polymorphism to provide a genetic linkage and ultimately to localize the gene itself. There will then be a real possibility of identifying the metabolic defect. □

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Rapid environmental and climatic changes

from D. Q. Bowen

THE successful characterization and evaluation of past rapid changes in the environment and climate depend largely on the integrity and sensitivity of dating methods. All too often imprecision in the climatic record stems from dating uncertainties, with bioturbation and a variety of lag effects providing additional problems. Given these constraints, it is little wonder that much of a recent meeting* on the character and timing of rapid environmental and climatic changes was devoted to the comparatively well-dated Holocene record and, to a lesser extent, to that of the later part of the last glaciation. Small-scale systems of generally short duration, but for which detailed data were available, received far more attention than the larger systems with somewhat coarser data available over longer time scales.

At the larger end of the scale it is difficult to identify specific climatic causes for changes in climate (R. V. Barry, University of Colorado, Boulder). Among the many uncertainties are whether fluctuations on the 100-1,000 year scale are due to longitudinal and/or latitudinal changes in circulation; whether particular regimes persist for long periods and what the circulation

variability of different regimes is. It is not even possible to ascribe the quasi-persistent types of circulation regime to external or internal forcings, to atmospheric auto-variation or to internal feedback mechanisms between land, ocean and atmosphere. Barry stressed, however, that the intensity and timing of climatic change will show considerable geographical variation: step-like in some areas but more gradual in others.

R. Bryson (University of Wisconsin, Madison) further stressed that in regarding climatic change as essentially nonlinear, a major break with tradition had now occurred; while J. Mitchell (Environmental Data Service, Silver Spring, Maryland) emphasized the role of the oceans as a global climatic fly-wheel which limits change more strictly over shorter than over longer periods. Deep water formation in the North Atlantic may also play an important part in speeding glacial events; sea-level changes can lead to perturbations in oceanic thermocline circulation processes which may enhance oceanic heat transportation to higher latitudes during periods of glacial growth (C. Rooth, University of Miami).

Global data show considerable glacial fluctuations during the last glaciation, mostly reflecting local or anomalous conditions (S. Porter, University of Washington, Seattle) and the glacier response to rapid climatic change is best assessed from Holocene examples where

lead and lag effects can be partially quantified. Sea-level data for the past 140 kyr (T. Cronin, US Geological Survey, Reston) demonstrate the many pressing problems still extant: where was the last glacial maximum sea level? Where was sea level during the mid-Wisconsin? How rapid were sea-level changes? The possibility of a high sea-level event between 130 and 140 kyr received much attention but was strongly questioned by W. Ruddiman (National Science Foundation) on the basis of the oxygen-18 record.

The most sensitive indications of climatic change are provided by the oxygen-18 record in ice sheets followed by sea-surface temperatures (H. Wright, University of Minnesota, Minneapolis). Most pollen records are beset by persistent difficulties in interpretation due to their proximity to ice sheets. Sensitivity and response times might thus be better detected at very low latitudes or in the Southern Hemisphere, free from such influences. Furthermore the climatic signal from pollen data fails to provide a clear record of climatic change of short duration even when its amplitude is large (M. Davis, University of Minnesota): for example, the 'Little Ice Age' is only recorded clearly by pollen at a few sites, despite the considerable impact on human culture. Except in a few cases it is difficult to infer from pollen data whether climatic change is step-wise or gradual and unidirectional (W. Watts, Trinity College Dublin). Relationships between climatic change and the pollen record are only indirect because plant populations generally become established long after causal climatic events. There is thus an urgent need to identify sensitive ecotonal areas at the margins of plant ranges where pollen data and climate can be linked directly. Most rapid of all may be the response of lake sediments to climatic change (R. Anderson, University of New Mexico, Albuquerque). Seasonal control is dominant and gives a clear signature, while cumulative changes in seasonal components demonstrate rapid climatic change. It should be possible to design transfer functions expressing linkages between climate and sedimentation, with eventual application to non-varied sediments.

In summing up the proceedings, J. Mitchell predicted that climate will change over a transitional period of the next 200 years because of an increased carbon dioxide content in the atmosphere, then settle into a 1,000 year equilibrium state. The rapidity of this transition could be comparable to that at the Younger Dryas to Holocene change some 10,000 years ago. □

Erratum

In K.F. Chater's article '*Streptomyces* in the ascendant' (*News and Views* **299**, 10; 2 September 1982), the last eleven lines of paragraph four (beginning 'As a result, ...') should have appeared at the end of paragraph five.

*The 7th Biennial Conference of the American Quaternary Association on 'Character and Timing of Rapid Environmental and Climatic Changes' was held at the University of Washington, Seattle, 28-30 June 1982.

D. Q. Bowen is in the Department of Geography, The University College of Wales, Aberystwyth SY23 3DB.

A Proterozoic Pangaea?

from D.H. Tarling

IN a recent review of palaeomagnetic data (*Earth planet. Sci. Lett.* **59**, 61; 1982), Piper concludes that a single huge continent, Pangaea, existed throughout the Proterozoic period of the Earth's history (2,600–570 Myr ago). Such a conclusion is consistent with many geological features that indicate extensive continental stability during this period, yet Piper's analyses also require that this Pangaeian supercontinent was in motion relative to the Earth's axis of rotation. Such motion must, presumably, be associated with the same mantle convective motion that currently results in continental splitting and ocean-floor spreading. Piper's conclusions are thus of fundamental importance in testing some of the basic assumptions in both palaeomagnetism and plate tectonics.

Palaeomagnetic studies allow the determination of the palaeolatitude and orientation (relative to the palaeomagnetic pole) of the sampled area. Where sufficient data are available, they can be used to construct a palaeomagnetic polar wandering curve, indicating the changing position of the pole relative to the stable tectonic block from which the samples were obtained. Such curves do not, in fact, mean that the pole is moving, as this is fixed relative to the ecliptic, but results from a movement of the sampled region relative to the pole. The fact that polar wandering curves exist for the Proterozoic is thus of the utmost importance as it clearly indicates that the continent, or continents, were in motion and were presumably driven by mantle convection.

If polar wandering paths for the same period of time can be matched between different tectonic units, then the relative positions of these tectonic units can be precisely defined. Thus the palaeomagnetic data from the different cratonic blocks of the world can be used to determine the existence, or not, of one or more continental units during the Proterozoic. Piper finds that he can define Pangaea throughout the Proterozoic as a cigar-shaped continent comprising Australia, China, India and Antarctica backing onto western South America–Africa, with Kazakhstan–Siberia against North Africa and bordering onto western Laurentia (North America, Greenland and Europe). Piper finds that such a continent is not only required by the palaeomagnetic data but is also consistent with the available geological evidence.

It is only too easy to be sceptical about the data base on which any such model is

erected. Even with a single craton, the radiometric dating of any intrusive event is uncertain within 100–200 Myr, even ignoring problems of open chemical systems, during which time major continental motions could have occurred. It is also worrying that much Precambrian data are based on alternating magnetic field demagnetizations — a technique that is 'noisier' than thermal demagnetization — yet thermal demagnetization is often apparently ineffective in determining discrete components of remanence. Even when such components can be isolated, there is doubt about their age. There is a tendency to assume that the component with the highest stability is, in fact, the oldest — yet this may simply reflect the presence of haematite that may have formed very much later than the rock itself. Even when such components have been isolated and dated, it is rare for there to be adequate tectonic control of subsequent movements, although the Proterozoic crust appears to have been little deformed as the overlying sediments tend to have only shallow dips. The uncertainties involved are thus immense and require subjective evaluation. Unfortunately, this

can lead to the syndrome in which scattered data are examined and the odd points that are near to previous observations are accepted for publication and the remainder appear, if at all, in the inaccessible appendix of a thesis. Despite Piper's attempts to be objective, therefore, the data may well already have been subjectively selected to conform to previous data or models. However, the only way in which such problems can be resolved is by the erection of specific models which can then be tested by a variety of methods and by new data.

This is not Piper's first model and it seems unlikely to be his last. However, the fact that he can make a very plausible case for support of his model is, in itself, of great importance. It demonstrates, for example, that continental motions occurred, thus indicating the action of mantle convection currents. This is no great surprise in view of the much higher radiogenic heat production in the past, but raises the problem of why such motions did not cause continental splitting, as they do now and also did in pre-Proterozoic times. The implication is that the continental lithosphere, in Proterozoic times, was too strong or too thick to be split by such motions. But how did such a thickness develop suddenly at the end of Archaean times and what caused the change to present-day continental splitting? □

The sunflower seed protection business

from Peter D. Moore

The consumption of the seeds of plants by invertebrate and vertebrate predators is a serious problem for the agronomist, and much effort has been devoted to finding ways of limiting the predators' numbers. There is, however, a quite different way of tackling the problem. In a recent paper¹, field trials are reported of attempts to protect economically important seeds by buying off the predators with an abundant supply of another seed.

In many North American forests, small mammals have often been found responsible for seed predation and depression of woodland regeneration, in particular the douglas fir (*Pseudotsuga menziesii*) has been much studied. That seed predators may limit the regeneration of plant species has been known for some time. As long ago as 1919 Watt's² records of the fate of acorns in oakwoods showed that high mortality of acorns in oakwoods could be largely put down to predation by birds and mammals. He also found that in the case of beech², invertebrates were involved and concluded that the low level of regeneration found in British woodland was a consequence of

seed predation. The high population density of mice and voles, regarded by him as major culprits, could in turn be related to rearing of game birds and consequent destruction of predators, a view supported and popularized by Tansley³.

The possibility that the problem might be overcome through providing an alternative food source was first approached experimentally by Sullivan⁴ who supplied deer mice (*Peromyscus maniculatus*, a major seed predator of douglas fir) with sunflower seed in order to distract it from the conifer seed. He found that very large quantities of sunflower seed had to be provided (seven sunflower seeds for every douglas fir seed) in order to reduce predation significantly.

Sullivan and Sullivan⁵ have now repeated these experiments with lodgepole pine (*Pine contorta*) in an area of British Columbia from which the pine had been

D.H. Tarling is Reader in Palaeomagnetism in the Department of Geophysics, The University, Newcastle upon Tyne NE1 7RU.

Peter D. Moore is Senior Lecturer in the Department of Plant Sciences at King's College, University of London.

felled in 1978. Lodgepole pine was seeded at a density of 45,000 ha⁻¹ and, in some experimental plots, sunflower seed was added at twice this density (90,000 ha⁻¹). Damage by mice was very high in plots without the sunflower additive (often less than 5 per cent survival after 5 months), but where sunflower seed was also sown, pine seed survival was around 25 per cent after 5 months. This fivefold increase in the rate of survival is obviously of great interest to the forester.

There are two complications, however, which lead to a certain degree of apprehension. The first, which has received emphasis from Harper⁶, is that compensating factors may be operating so that when one cause of mortality is reduced another assumes prominence. The second is the fear that a rich new resource of sunflower seeds may cause an increase in mouse population density, which will subsequently lead to greater predation upon the pine seeds. Sullivan and Sullivan claim that such increases in mouse density were observed in the sunflower plots only where initial densities were below 5 mice ha⁻¹, so other population limits are evidently in operation. It also appears from their data that no other cause of pine seed mortality has replaced that due to predation, so the technique seems to warrant more extensive trials.

Ecological, or perhaps ethical, attitudes have evidently changed since the days of Watt and Tansley. Instead of shooting the predators we now feed the mice. □

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Glomar Challenger at the Cretaceous-Tertiary boundary

from Audrey Wright, Ross Heath and Lloyd Burckle

THE drilling vessel *Glomar Challenger* reached Yokohama on 20 June 1982, after sampling six sites in the western North Pacific. Major objectives of this leg were to determine the chronology and extent of late Neogene through Pleistocene migrations of the subarctic front; to acquire a Cenozoic record of aeolian and authigenic sedimentation in the North Pacific; and to collect an undisturbed sample of the Cretaceous-Tertiary boundary on Shatsky Rise.

Hydraulic piston cores taken at four sites located along a north-south transect from 34° to 44°N latitude and centred at about 155°E longitude recovered a suite of siliceous sediments for in-depth palaeoceanographic and palaeoclimatic reconstructions for the past five million years. Sediments from two 'red clay' sites, located east and west of Shatsky Rise, provide an excellent basis for resolving Cenozoic North Pacific authigenic sedimentation as well as for detailing the depositional history of wind-blown sediment from Asia.

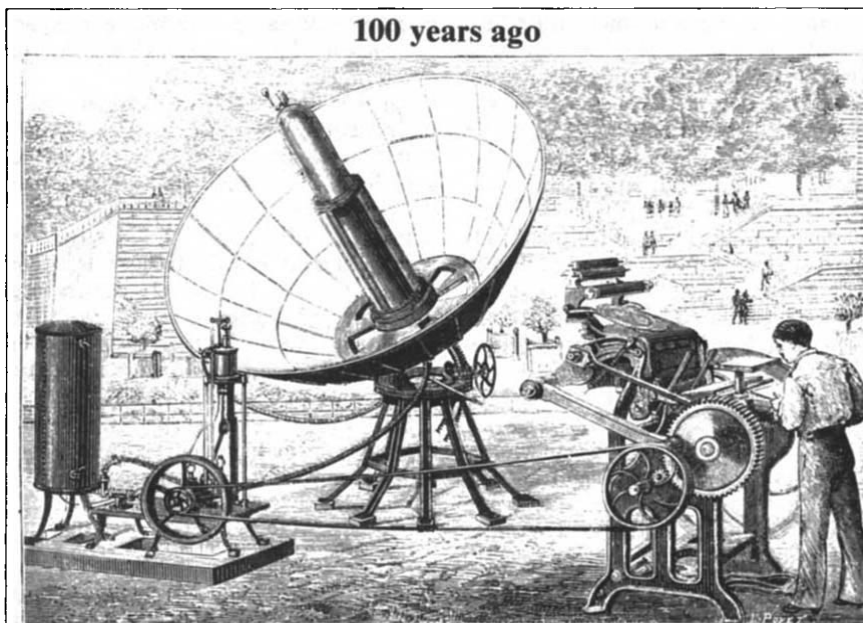
At Site 577 on the Shatsky Rise, a complete and expanded sequence containing the Cretaceous-Tertiary (K-T) boundary was collected. Triplicate hydraulic piston cores were successfully taken through this critical interval, guaranteeing complete coverage and sufficient material for detailed study of floral and faunal changes as well as palaeomagnetic and geochemical properties across the K-T boundary. Studies of the foraminifers, nannofossils, palaeomagnetic signature and aeolian debris from this section, begun at sea, will be continued

on-shore in the laboratories of cruise participants.

Initial examinations of the calcareous nannofossils and planktonic foraminifers suggest that the K-T boundary, located at a sub-bottom depth of 109.7 m, is continuous and complete. The abundance of the late Maestrichtian *Micula mura* nannofossil assemblage decreases abruptly at the boundary. Above it, Cretaceous species are replaced by a less diverse, less abundant and moderately preserved assemblage characterized by the genera *Thoracosphaera* and *Cyclagelosphaera*, as well as *Markalius astroporus* and the very small *Cruciplacolithus primus*. Few reworked Cretaceous forms were found 20 cm above the boundary.

Preservation of foraminifers is moderate to good throughout the boundary interval. The K-T boundary is marked by the extinction of all Cretaceous planktonic forms and the first appearance of the early Palaeocene foraminifer *Chiloguembelina taurica*. Rare reworked Cretaceous foraminifers are found at least 20 cm above the boundary. The first appearance of the primitive globigerinid foraminifer, *Globigerina eugubina*, occurs 40 cm below the boundary. At this point the specimens are few in number and very tiny. They become abundant and of a consistently larger size at the boundary. These preliminary findings are in contrast with the section at Gubbio, Italy, in which the first appearance of *G. eugubina* marks the K-T boundary. □

Audrey Wright, Ross Heath and Lloyd Burckle are members of the DSDP 86 scientific staff.



A SOLAR PRINTING PRESS

It was mentioned in a recent number of this journal that a printing press worked by solar heat had been exhibited in the Tuileries Garden in Paris on the occasion of a *fête*. The solar generator was devised by M. Abel Pifre. The insulator, shown in the middle of the picture, measured 3.50 m. diameter at the aperture of the parabolic mirror. It was set up in the garden, near the large basin, at the foot of the flight of steps of the Jeu de Paume. The steam from the boiler placed in its focus was utilised by means of a small vertical motor (shown on the left), having a power of 30 kilogrammetres, which actuated a Marinoni press (on the right). Though the sun was not very ardent, and the radiation was hindered by frequent clouds, the press was worked with regularity from 1 p.m. till 5.30 p.m., printing on an average 500 copies an hour, of a journal specially composed for the occasion, viz., the *Soleil Journal*. This result, though not indicating a revolution in the art of printing, may enable one to judge of the services these *insulators* may render in climates with a radiation more powerful and constant. From *Nature* 26, 503; September 21, 1882.

ARTICLES

Sharp edges of planetary rings

Nicole Borderies & Peter Goldreich

Division of Geological and Planetary Sciences, California Institute of Technology, Pasadena, California 91125, USA

Scott Tremaine

Department of Physics, Center for Space Research and Center for Theoretical Physics, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

The ring systems of Saturn and Uranus exhibit several sharp edges across which the optical depth drops from order unity to essentially zero. At least two and perhaps all of these features are associated with the location of orbital resonances between a satellite and the ring particles. It is remarkable that the optical depth varies on a distance scale which is much finer than that over which angular momentum can be transferred between a satellite and the ring material. The important features of this phenomenon are: (1) A perturbed band of width $\Delta a/a \approx (M_s/M_p)^{1/2}$ adjacent to the edge within which the angular momentum transfer occurs. (2) Streamlines perturbed such that the angular momentum luminosity decreases smoothly across the band to zero at the edge even though the optical depth remains constant. (3) Dynamical equilibrium requires a relation between the random velocity, the rate of deformation and the optical depth.

SHARP edges are common features of planetary rings¹. Stellar occultation observations of the rings of Uranus revealed edges which are sharp at a resolution of a few kilometres². Stellar and radio occultations carried out by the Voyager spacecraft revealed several sharply defined gaps and ringlets in the saturnian ring system (refs 3, 4 and L. Tyler, personal communication). Of these, perhaps the most interesting are the outer boundaries of the classical A and B rings which are sharp on a scale of hundreds of metres. Voyager discovered that the outer boundary of the A ring is virtually coincident with the 7:6 resonance of the larger co-orbital satellite⁴ and confirmed the prediction that the outer boundary of the B ring is located at the 2:1 resonance with Mimas^{5,6}. On the basis of these two examples, we adopt the hypothesis that a satellite resonance is associated with every sharp edge across which the optical depth falls to zero.

Here we consider the dynamics in several stages. First, we review some relevant material about periodic orbits and particle disks, and then we prove that the ring particle velocity dispersion is greatly enhanced near a sharp edge. Next, we show how the angular momentum luminosity gradually decays to zero even though the surface density and velocity dispersion do not vary appreciably. Finally, we indicate why the surface density remains high until it drops abruptly to zero over a distance from the edge comparable to the vertical thickness of the ring.

The semi-quantitative arguments presented here are, in part, based on numerical calculations of ring equilibria near an edge, the results of which we shall publish elsewhere. For ease of notation, and because the outer A and B ring boundaries are among the best studied examples of sharp edges, we have concerned ourselves only with outer edges; the discussion can be translated easily to render it applicable to inner edges.

Periodic orbits

The perturbation potential near an isolated resonance is characterized by an azimuthal wavenumber m and a pattern speed Ω_p . In a reference frame which rotates with the pattern speed, the potential varies as $\cos m\phi$ with longitude ϕ .

There is a set of test particle orbits, referred to as periodic orbits, which are closed in the rotating frame. In the inertial frame the periodic orbits are precessing ellipses. For small

eccentricity, the shapes of the orbits in the rotating frame satisfy

$$r = a \left[1 + \Gamma \frac{M_s a}{M_p (a - a_r)} \cos m\phi \right] \quad (1)$$

where r is the radial distance from the centre of the planet, a is the semi-major axis, M_s and M_p are the masses of the satellite and planet, respectively, and Γ is of order unity. The eccentricity increases with decreasing distance from the resonance which is at semi-major axes a_r . In a region of width

$$\Delta a \approx a_r (M_s/M_p)^{1/2} \quad (2)$$

around a_r , the periodic orbits intersect.

Streamlines

The ring particle streamlines are described by

$$r = a \{ 1 - e(a) \cos m[\phi + \Delta(a)] \} \quad (3)$$

in the rotating frame. Here a and e are the semi-major axis and eccentricity of the ring particle orbits and Δ is a phase lag. The determination of e and Δ from the viscous stress and gravitational perturbations due to the satellite and the ring material is described later. The outer edge a_0 is near a , and for $(a_0 - a)/\Delta a \gg 1$, the streamlines are well approximated by periodic orbits. Closer to a_0 , the self-gravity of the ring material and the interparticle stress modify the streamlines so that they do not cross.

For later use, we define

$$J(a, \phi) = \frac{\partial r}{\partial a} \bigg|_{\phi} = 1 - q \cos [m(\phi + \Delta) + \gamma] \quad (4)$$

where

$$q^2 = \left(\frac{a de}{da} \right)^2 + \left(\frac{mae d\Delta}{da} \right)^2, \quad \text{sgn } q = \text{sgn} \left(\frac{de}{da} \right) \quad (5)$$

and

$$\tan \gamma = \frac{me d\Delta/da}{de/da}, \quad -\frac{\pi}{2} \leq \gamma \leq \frac{\pi}{2} \quad (6)$$

Viscosity

A rigorous treatment of the viscous stress in a particle disk of modest optical depth requires the derivation of the full stress tensor from kinetic theory. The Boltzmann equation with col-

lisions has been solved for perturbed particle disks⁷. We rely on this calculation for guidance but for simplicity we use the hydrodynamical approximation and relate the viscous stress to the deformation rate tensor by a single parameter, the kinematic viscosity, ν .

For an unperturbed keplerian disk it is a good approximation to take

$$\nu = 0.5 \frac{v^2 \tau}{\Omega(1 + \tau^2)} \quad (7)$$

where v is the one-dimensional r.m.s. random velocity⁸. For simplicity, we ignore the real complications introduced by anisotropic random velocities and the distribution of particle sizes. It is qualitatively acceptable but quantitatively inaccurate to extend the use of equation (7) to regions perturbed by resonances.

The rate of dissipation of mechanical energy per unit mass is expressed macroscopically by

$$\frac{dE_v}{dt} = 2\nu |S|^2 \quad (8)$$

and microscopically by

$$\frac{dE_v}{dt} = (1 - \varepsilon^2) v^2 \Omega \tau \quad (9)$$

Here S is the rate of deformation tensor and ε is the coefficient of restitution for particle collisions. Equating the two expressions for viscous dissipation and using equation (7) for ν , we obtain

$$\varepsilon^2 \approx 1 - \frac{G(q^2)}{1 + \tau^2} \quad (10)$$

where

$$G(q^2) \approx |S/\Omega|^2 \quad (11)$$

If $\varepsilon(v)$ were known, equation (10) would determine v as a function of q^2 and τ . Unfortunately, we can be confident only that $d\varepsilon/dv < 0$, $dG/d|q| > 0$ and $G \rightarrow \infty$ as $|q| \rightarrow 1$. However, we can draw one important conclusion from equation (10): that is, in steady state the magnitude of the rate of deformation is bounded such that

$$|S| \leq \Omega(1 + \tau^2)^{1/2} \quad (12)$$

An external stress might temporarily drive $|S|$ above the upper limit. However, the random velocity would then grow exponentially, with rate constant $\Omega\tau$, until the viscous stress was large enough to reduce $|S|$ to an acceptable steady-state level.

Angular momentum and energy fluxes

The angular momentum and energy fluxes, $F_H(a, \varphi)$ and $F_E(a, \varphi)$, are the rates at which these quantities cross a unit length of the streamline with semi-major axis a and longitude φ . The luminosities $L_H(a)$ and $L_E(a)$ are the integrals of the fluxes around the streamline.

The ring divides naturally into two regions, an inner unperturbed portion with $a < a_1$ and an outer perturbed part with $a_1 \leq a \leq a_0$. The location of a_1 need not be specified beyond the requirement that $\Delta a \ll a_0 - a_1 \ll a_r$.

Unperturbed inner region. In the inner, or keplerian, region the streamlines are circles, $\Omega^2 a^3 = GM_p$, and

$$L_H(a) = 2\pi a F_H(a) = 3\pi \Sigma \nu \Omega a^2 \quad (13)$$

$$L_E(a) = \Omega L_H(a) \quad (14)$$

with Σ the surface density⁹. The radial drift velocity of a ringlet is

$$\frac{da}{dt} = -\frac{1}{\pi a^2 \Omega \Sigma} \frac{dL_H}{da} \quad (15)$$

and vanishes if L_H is conserved. The viscous dissipation of energy per unit mass is

$$\frac{dE_v}{dt} = -\frac{1}{2\pi a \Sigma} \left[\frac{dL_E}{da} - \frac{\Omega dL_H}{da} \right] = \frac{3\Omega L_H}{4\pi a^2 \Sigma} = \frac{9\nu \Omega^2}{4} \quad (16)$$

Note that L_E decreases outwards even where L_H is conserved. **Perturbed outer region.** Here the streamlines are not circular and the fluxes are functions of φ as well as of a . The hydrodynamical approximation yields

$$F_H(a, \varphi) = 2\nu(a) \Sigma(a) \Omega a (1 - q^2)^{1/2} \left[\frac{1}{J} - \frac{1}{4J^2} \right] \quad (17)$$

where $\Sigma(a)$ is the mean surface density averaged over φ and $\Omega(a)$ is the mean orbital angular velocity. As $e(a) \ll 1$, $F_E = \Omega F_H$. If ν is assumed to be a function of a but not of φ , then

$$L_H(a) = 4\pi \nu \Sigma \Omega a^2 K(q^2) \quad (18)$$

where

$$K(q^2) = \frac{3 - 4q^2}{4(1 - q^2)} \quad (19)$$

Of course, $L_E = \Omega L_H$.

Two values of $|q|$ are of particular significance. For $|q| = q_1 = 3/4$, $F_H = 0$ where $\cos[m(\varphi + \Delta) + \gamma] = \text{sgn } q$. For $|q| < q_1$, $F_H > 0$ for all φ and for $|q| > q_1$, there is an interval of φ in which $F_H < 0$. The inward flux of angular momentum arises because the angular velocity increases outwards in this longitude range. For $|q| = q_2 = \sqrt{3}/2$, $L_H = 0$. For $|q| < q_2$, $L_H > 0$ and for $|q| > q_2$, $L_H < 0$.

The existence of two transitional values of $|q|$ is established by our solution of the Boltzmann equation⁷. The particular numerical values, $q_1 = 3/4$ and $q_2 = \sqrt{3}/2$, are special consequences of the hydrodynamical approximation. The more appropriate kinetic theory yields values which are functions of optical depth.

Conditions near edges

Enhanced velocity dispersion. An outer edge may remain fixed at a resonance if the angular momentum which flows out from the unperturbed inner region, $L_H(a_1)$, is completely absorbed by the satellite. Then, it follows from the Jacobi relation that energy is transferred to the satellite at a rate $\Omega_p L_H(a_1)$. The rate of energy dissipation in the perturbed region must be $L_E(a_1) - \Omega_p L_H(a_1) = (\Omega - \Omega_p) L_H(a_1)$. Most of this dissipation takes place in the narrow outer band of width Δa in which the streamlines are severely distorted. The average dissipation rate per unit mass in this band is

$$\frac{dE_v}{dt} = \frac{(\Omega - \Omega_p)}{2\pi a \Delta a \Sigma} L_H(a_1) \quad (20)$$

Comparison of equations (16) and (20) shows that dE_v/dt is enhanced in the outer band by a factor $2a(\Omega - \Omega_p)/(3\Omega \Delta a)$ relative to its unperturbed value. Use of the resonance condition $m(\Omega - \Omega_p) = \Omega$ and $\Delta a/a \approx (M_s/M_p)^{1/2}$ allows us to simplify the enhancement factor to $m^{-1}(M_p/M_s)^{1/2}$.

It is apparent from equation (8) that either or both ν and $|S|$ must increase in the outer band. However, the upper limit on $|S|$ given by equation (12) proves that for $\tau = 0(1)$, most of the enhanced dissipation is due to an increased ν . Since ν is proportional to v^2 , it follows that v is enhanced by a factor

$$F \approx \left[\frac{1}{m^2} \frac{M_p}{M_s} \right]^{1/4} \quad (21)$$

Decay of L_H . It might seem peculiar that L_H would decay in the outer band where Σ is constant and ν is enhanced above its unperturbed value. However, there is no paradox here.

$L_H = 0$ for any values of Σ and ν if $|q| = q_2$. Thus $q_2 - |q| \ll 1$ in the outer band and $|q| = q_2$ at the edge.

Sharp edges. We have seen how L_H can decay to zero in the outer band at constant Σ . However, one question remains: why does Σ maintain a constant value right up to the edge?

To answer this, we refer to equation (10) which relates ϵ , τ and $|q|$. Since ϵ is a function (admittedly unknown) of ν , and τ is directly proportional to Σ , equation (10) may be viewed as connecting ν , Σ and $|q|$. But in the outer band, $|q| = q_2$ and ν is enhanced by a factor F above its unperturbed value. Thus, in principle, equation (10) determines Σ in the outer band as a function of the resonance parameter $m^{-1}(M_p/M_s)^{1/2}$ and the unperturbed ν .

The precise distance over which Σ drops to zero is probably of the order ν/Ω which is the vertical thickness of the ring and, for $\tau = 0(1)$, is also the mean free path for particle collisions. Our use of the hydrodynamical model is not valid closer than ν/Ω to the ring edge.

Numerical results

The dynamical state of the ring may be modelled by dividing it into narrow ringlets whose shapes are determined by the three variables a , e and Δ . The perturbation accelerations are due to the satellite gravity, the self-gravity of the ring and the viscous stress. We have derived the differential equations which govern the variations of a , e and Δ for each ringlet. The evolution equations are supplemented by equations (7) and (10) and one of several plausible relations for $\nu(\epsilon)$.

Equilibrium solutions have been obtained with up to 20 ringlets. They exhibit the features discussed above. In particular, there is an outer band of width approximately Δa in which ν is greatly enhanced, $|q| = q_2$, Σ is fairly constant and L_H decays to zero. With some forms of $\epsilon(\nu)$ we were unable to obtain equilibrium solutions for which Σ varies smoothly with a in the outer band. Presumably, the smooth equilibria suffer from a secular instability of the kind proposed to explain the multiple ringlet structure of Saturn's rings¹⁰⁻¹².

Single ringlet model for the outer band. Several characteristics of the numerical solutions may be illustrated by a crude model in which the entire outer band is represented by a single ringlet.

We balance the satellite torque on the ringlet

$$T_s \approx -(ma\Omega)^2 e \frac{M_s}{M_p} \Sigma a \Delta a |\sin m\Delta| \quad (22)$$

against the angular momentum luminosity which enters it, $L_H(a_1)$, to obtain

$$|\sin m\Delta| \approx \frac{\nu}{\Omega(ma)^2} \left(\frac{M_p}{M_s} \right)^2 \quad (23)$$

where ν is the unperturbed viscosity. In deriving equation (23) we have set $e = \Delta a/a \approx (M_s/M_p)^{1/2}$. The condition that the satellite is capable of opening a gap at the resonance is equivalent to $|\sin m\Delta| \leq 1$ (ref. 13).

The values a_0 and e_0 for the outer edge depend on the parameters m , M_s/M_p and $\Sigma a^2/M_p$. At a strong resonance,

$|\sin m\Delta| \ll 1$ so that $q \approx a \, de/da$. Now $q = |q_2|$ in the outer band and, for our hydrodynamical mode, $q_2 = \sqrt{3}/2$. The expression

$$e \approx \left(\frac{M_s}{M_p} \right)^{1/2} \left[1 + \frac{\sqrt{3}}{2} \right] + \frac{\sqrt{3}}{2} \left[\frac{a - a_r}{a_r} \right] \quad (24)$$

takes into account the fact that the streamlines are well approximated by periodic orbits for $a < a_r - \Delta a$ and that $a \, de/da \approx \sqrt{3}/2$ for $a \geq a_r - \Delta a$. The condition that the outer edge streamline does not precess in the rotating frame is

$$\frac{\Sigma a^2}{M_p e_0} - \frac{m M_s}{e_0 M_p} \approx \frac{3(m-1)}{2} \left[\frac{a_0 - a_r}{a_r} \right] \quad (25)$$

The first and second terms on the left-hand side are due to the gravitational perturbations from the ring and the satellite, which force the apsidal line to advance and regress, respectively. For $p \approx \Sigma a^2/(m M_s) \gg 0(1)$, equations (24) and (25) may be solved for $(a_0 - a_r)/a$ and e_0 . Both e_0 and $(a_0 - a_r)/a$ are proportional to $(p M_s/M_p)^{1/2}$ for $p \gg 1$.

Discussion

The Voyager 2 images show that the outer boundary of the B ring is an ellipse centred on Saturn whose short axis points approximately towards Mimas³. A calculation of the periodic orbits near the 2:1 resonance with Mimas indicates that the first orbits which cross have $ae = 30$ km. The observations yield $ae \approx 70$ km for the B-ring outer edge. This information may allow a local determination of Σ from more precise versions of equations (24) and (25).

The velocity dispersion and the vertical thickness should be enhanced by between one and two orders of magnitude in the outer band of the B ring relative to their unperturbed values. It is reasonable to assume that the enhanced collision velocities associated with resonance features such as sharp edges and density wavetrains would result in the copious production of small particles. These particles might account for the peaks in optical depth found near resonances in the UV spectrometry occultation data⁴. These peaks should then be absent from the radio occultation data.

The arguments presented in this article are based on the implicit assumption that outside a sharp edge $L_H = 0$, or at least is reduced far below its value in the unperturbed part of the ring. The possibility remains that although τ drops by one or even two orders of magnitude, ν might rise by a similar factor, thus maintaining L_H at its unperturbed value in a region which is apparently empty. The juxtaposition of high and low optical depth regions separated by a sharp boundary would then be an extreme limit of the secular instability proposed to account for the fine structure in Saturn's rings¹⁰⁻¹².

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Stratigraphical significance of the Tulu Bor Tuff of the Koobi Fora Formation

F. H. Brown & T. E. Cerling

Department of Geology, University of Utah, Salt Lake City, Utah 84112, USA

The Tulu Bor Tuff, a major marker horizon in the Koobi Fora Formation, has been misidentified in previous studies. Identification of this tuff in sediments previously mapped as the Kubi Algi Formation requires re-evaluation of the existing stratigraphical framework of the Koobi Fora region.

DURING the past 2 yr we have investigated the chemistry of tuffs from Plio-Pleistocene deposits east of Lake Turkana in northern Kenya. The stratigraphy at Koobi Fora is based on marker beds and their supposed correlatives¹. We state specific locations where particular stratigraphical marker beds may be observed. This is necessary because miscorrelations have been made in erecting the type sections of formally defined formations, and because several stratigraphical terms now in use are defined only vaguely. In addition, we find that considerable stratigraphical revision is needed at Koobi Fora.

Stratigraphical problems at Koobi Fora have been evident for some time. By 1977 it had been shown² that chemical differences between the Tulu Bor Tuff in area 129 and what had been called the Tulu Bor Tuff in areas 15, 102 and 116 were significant. Identification of two distinct tuffs which had been previously mapped as the KBS Tuff also indicated that some miscorrelations had been made^{2,3}. Also in 1977 White and Harris⁴ stated that "suids from the lower Member of the Koobi Fora Formation (below ... the Tulu Bor Tuff) equate well with those from Member B. ... The Koobi Fora Formation lacks fossiliferous horizons equivalent in age to Shungura Members C through F, although there are indications that the Kubi Algi fauna may, in part, fill this hiatus", implying the Kubi Algi Formation must be partly equivalent to the Koobi Fora Formation^{5,6}. In 1981 Williamson⁷ defined 10 molluscan range zones in the type sections of the Kubi Algi, Koobi Fora and Guomde Formation must be partly equivalent to the Koobi Fora Formation^{5,6}. In 1981 Williamson⁷ defined 10 molluscan range zones horizons had been called the Tulu Bor Tuff and advocated substantial stratigraphical revision of the Kubi Algi and Koobi Fora Formations. Despite these disturbing observations, the same stratigraphical sections were published⁸ in 1981 as were published in 1973, with no comments about possible problems.

Stratigraphy and stratigraphical nomenclature

The stratigraphy of the Koobi Fora region has been widely discussed^{1,2,4,7-18}. In a reconnaissance study from 1970 to 1973 Bowen^{1,12} recognized three Plio-Pleistocene lithological units, the Kubi Algi Formation, the Koobi Fora Formation and the Guomde Formation. Only the Koobi Fora Formation was studied in detail, and was divided into the Upper, Lower and Ileret Members. Three stratigraphical levels were mapped during this study: the Suregei Tuff, the transition between the laminated siltstone facies and the facies above it (near the level of the KBS Tuff), and the Chari Tuff where it is overlain by the Guomde Formation¹²⁻¹⁴. Detailed mapping of the region followed (1972-80), and many additional marker horizons were recognized by Findlater¹⁵⁻¹⁸ and mapped throughout the region. In many cases the horizons mapped by Bowen were mapped differently by Findlater, but no reasons were stated for the changes.

In 1977 White and Harris⁴ published a tuff indexing system, and recorded the type areas of the named marker beds. Vondra and Bowen¹⁴ eliminated the Ileret Member in 1978, and redefined the lower boundary of the Upper Member of the

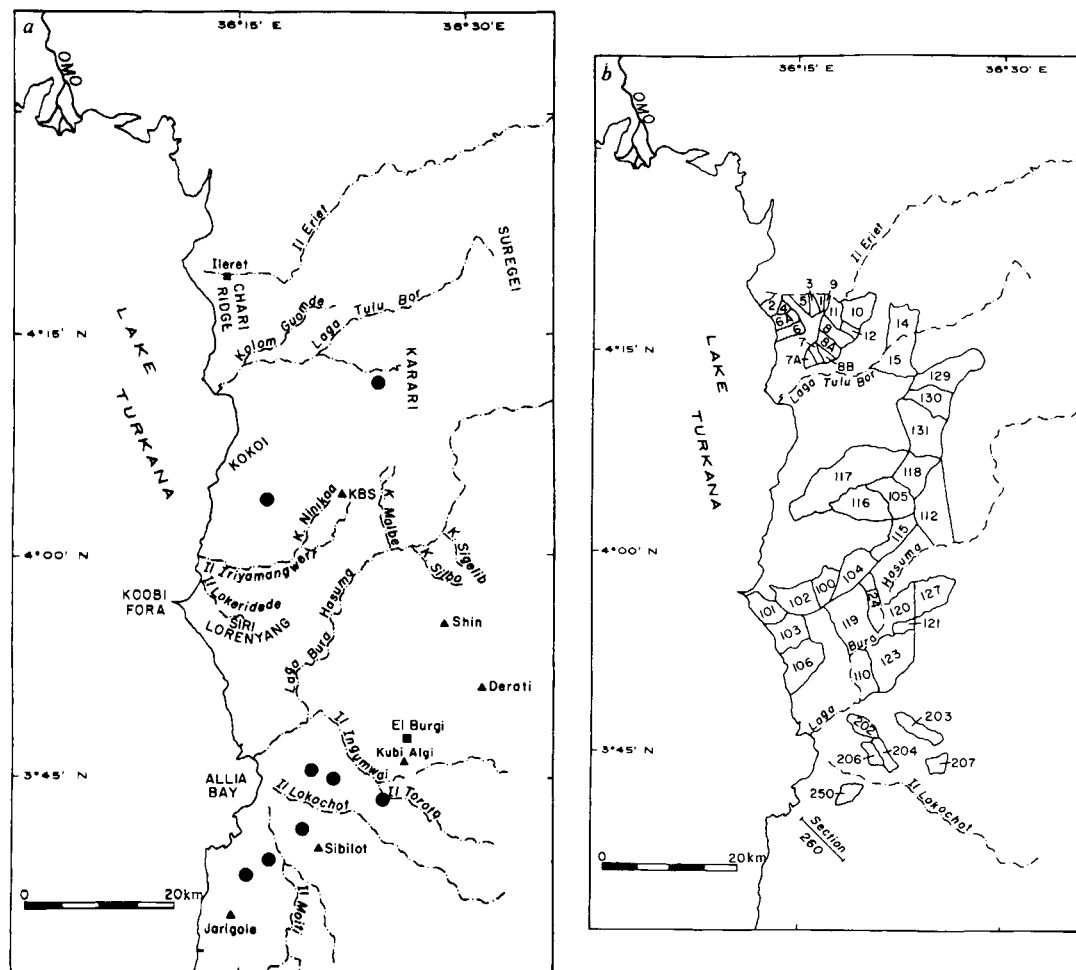
Koobi Fora Formation as the first channel conglomerate above the KBS Tuff.

The three Plio-Pleistocene formations were defined using the Suregei, Tulu Bor, KBS and Chari Tuffs either as defining horizons or as horizons useful for correlation from one part of the basin to another. The Kubi Algi Formation (the oldest) was defined as "those strata that lie below the basal contact of the Suregei Tuff Complex"¹. The Koobi Fora Formation was defined as "those sedimentary strata which lie between the basal contact of the Suregei Tuff Complex and the upper contact of the prominent tuff exposed along the Ileret ridge, here named the Chari Tuff, or the basal contact of the Holocene gray tuffaceous predominantly lacustrine siltstones which represent a late stage rise in the level of Lake Rudolf"¹. The Guomde Formation was defined as "those strata which lie between the top of the Chari Tuff on the Ileret ridge and the overlying gray tuffaceous siltstones which represent a late stage rise in the lake level"¹. To understand clearly what is meant by the stratigraphy, we must determine what was meant by each of the marker beds when the formations were defined.

Suregei Tuff Complex: What was originally referred to as the Suregei Tuff Complex has recently been called the Suregei Diatomite Complex⁸ following demonstration that the Suregei Tuff Complex is not tuffaceous². Most of the diatoms in this unit are recrystallized, however, and therefore we refer to this unit as the Suregei Complex. Bowen and Vondra¹ state that

Table 1 Analyses of the Tulu Bor Tuff, and of tuffs thought to be the Tulu Bor Tuff

Area	Sample no.	Fe ₂ O ₃	CaO	Ba	Mn	Nb	Ti	Y	Zn	Zr
117	80-183	1.51	0.65	491	330	70	1,533	49	58	380
129	77-26	1.54	0.66	480	336	68	1,513	50	70	388
129	77-27	1.46	0.61	452	326	70	1,452	50	55	388
129	80-212	1.37	0.58	448	287	68	1,378	50	75	328
129	77-24	1.25	0.61	427	240	72	1,437	52	53	280
s. 260	80-288	1.31	0.58	419	274	77	1,482	54	57	298
s. 260	80-289	1.29	0.50	419	265	74	1,319	55	55	276
s. 260	80-290	1.25	0.47	407	250	77	1,250	54	62	280
204	81-635	1.26	0.48	407	250	77	1,185	53	56	272
204	81-636	1.29	0.49	406	275	76	1,202	55	59	290
117	75-117C	1.55	0.40	152	364	82	940	61	74	349
207	81-460	1.45	0.33	143	372	81	925	61	87	351
117	80-182	1.52	0.33	140	372	84	975	62	73	373
117	80-179	1.56	0.32	135	387	83	930	63	79	366
117	80-181	1.55	0.31	135	381	81	925	63	88	360
117	80-177	1.56	0.31	134	392	85	962	63	73	386
117	80-778	1.52	0.32	130	362	84	1,045	62	79	389
204	81-437	1.46	0.30	122	351	85	877	62	68	357
102	72-38	2.36	0.16	9	1,408	154	1,012	84	149	809
102	80-104	2.20	0.15	5	1,448	156	900	84	153	823
102	80-123	2.07	0.15	5	1,408	154	898	85	150	807
110	77-32	3.16	0.20	26	881	201	1,197	130	243	1,284
116	75-116LT	4.05	0.39	285	1,889	129	3,725	72	173	784
116	75-116DK	4.27	0.19	448	1,757	109	4,477	57	153	626
15	72-188	3.23	0.41	182	1,169	132	2,444	79	140	982
15	80-136	2.53	1.13	464	1,075	119	3,119	77	111	825



Bowen and Vondra¹ correlated three marker beds from the northern part of the region to other parts of the basin on the basis of internal stratigraphical consistency. From lowest to highest these levels were: (1) the Suregei Tuff, recognized by its white colour and fine grained nature, and by the fact that the enclosing sediments were silty claystones; (2) the Tulu Bor Tuff, recognized by its distance 80 m above the Suregei Tuff and by its 3 m thickness; and (3) the KBS Tuff, recognized by its 30 m distance above the Tulu Bor Tuff, its fluvial nature, its position several metres above the highest beds of laminated siltstone facies, and its 'salt-and-pepper' appearance. These horizons were originally recognized in the northern part of the Koobi Fora region; later three similar horizons in area 102 were correlated with them. Additional support for the correlation of the Tulu Bor Tuff from area 129 to area 102 was provided by oxygen isotope analyses²⁰, although other proposed correlations were negated by the same data.

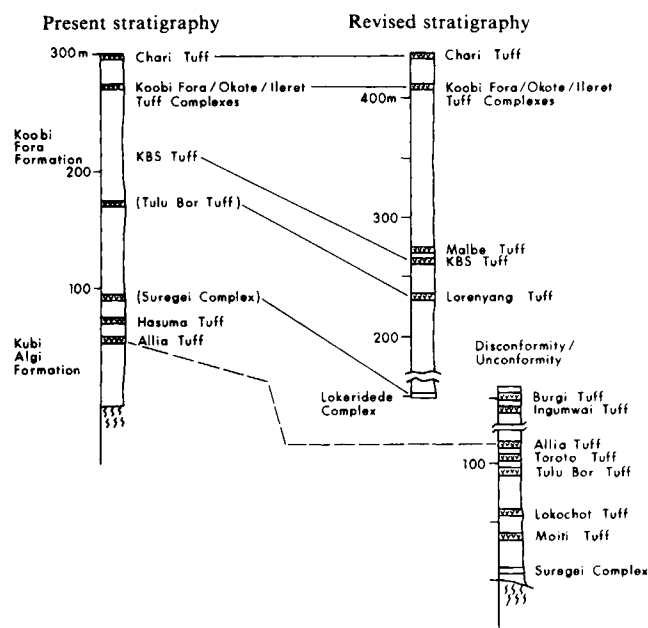


Fig. 2 Comparison of existing and revised stratigraphical nomenclature of Plio-Pleistocene sediments of the Koobi Fora region. See ref. 19 for additional discussion.

Our approach to stratigraphical correlation in the Koobi Fora region is based on chemical characterization of the tuffs, a method used successfully in many other studies²¹⁻²³. In many cases tuffs of the Koobi Fora region are very impure, sometimes consisting of only 10–20% glass shards. Bulk analyses yield meaningless results for purposes of correlation, and early attempts at using this method were discouraging²⁴. For this reason, we produced glass separates from the tuffs using heavy liquids and magnetic separation; calcite was removed with 10% HNO₃; clay films and limonitic staining were removed with 5% HF in conjunction with ultrasonic vibration. Each sample was checked optically to ensure that the separates consisted of >99% glass before analysis. In general the only remaining impurities in the glass were minute feldspar microlites in the shards.

The samples were analysed by X-ray fluorescence techniques on pressed pellets of the glass mixed with cellulose in a 4:1 ratio. Because these tuffs are alkaline or possibly peralkaline²⁵, they are rather different in trace element chemistry from most standard rocks. Two samples were analysed by the standard addition method, and were then used as standards for determination of Nb, Zr, Y, Sr, Rb and Zn on all other samples. Absorption corrections for these elements were made using the reciprocal of background. Analyses for Ti and Ba were made using United States Geological Survey standard G-2; correction for absorption was made using a regression based on potassium content. Iron and manganese were determined colorimetrically on a series of samples spanning the total range of concentration observed; other samples were analysed by X-ray fluorescence for these elements using the colorimetrically analysed samples as standards. Absorption corrections for iron and manganese were estimated by determination of absorption of CrK β . Precision and accuracy for all elements except barium are estimated to be ~2% and 5%, respectively; barium values are within 10% except those below 100 p.p.m., when the values are ± 25 p.p.m.

Our method of correlation depends primarily on the ability to recognize tuffs which are chemically distinct, rather than on the character of the sediments which enclose the tuffs. We have described problems which might be encountered in this method of correlation in a separate paper²⁶. Here only analyses of the Tulu Bor Tuff and tuffs formerly correlated with the Tulu Bor Tuff are presented (Table 1). However, analyses of more than

200 samples of some 30 distinct tuffs from the region are consistent with the correlations proposed below, and none of our correlations are inconsistent with correlations which might be made from stratigraphical considerations, although they differ from those proposed by earlier workers.

Identification of the Tulu Bor Tuff does not rest on analyses of that unit alone. In both the northern and southern part of the region the Lokochot Tuff¹⁹ underlies the Tulu Bor Tuff. The Tulu Bor Tuff and the Lokochot Tuff are correlative with Tuffs B and A of the Shungura Formation, respectively¹⁹.

Distribution of the Tulu Bor Tuff

We have analysed the Tulu Bor Tuff in area 129, its type locality, in area 117, and in areas 204, 207 and section 260 where it had not been previously recognized. We have also analysed samples of what was said to be the Tulu Bor Tuff in areas 15, 102, 110 and 116. The tuffs in areas 15, 102, 110 and 116 which were thought to be the Tulu Bor are not.

The tuff described as the Tulu Bor Tuff in area 102 is here named the Lorenyang Tuff after the local name for part of the Koobi Fora Ridge (Siri Lorenyang). The type locality of this tuff is in area 102, where it is best exposed in the centre of an anticline located in the north central part of the area. Here it is 3 m thick, is grey, fine grained, contains disrupted small scale cross-beds and has a sharp basal contact with a gradational upper one. The shards are frothy and quite distinct from the flat shards of the Tulu Bor Tuff. It also occurs in area 104, where it had been mapped¹⁸ as the KBS Tuff.

The tuff previously mapped as the Tulu Bor Tuff in area 110 is the KBS Tuff. This is sensible as the section above it is very similar to the section above the KBS Tuff in area 102.

The tuff in area 116 is the source of material dated at 3.18 Myr (ref. 27), a number generally associated with the Tulu Bor Tuff. This tuff is the uppermost of three tuffs in a continuous section ~10 m thick, and is here designated the Ninikaa Tuff. It consists of medium to fine cross-bedded grey ash and contains pumice clasts up to 20 cm in diameter. About 30% of the shards are brown, and shard shapes include bubble junction, stretched and frothy types. It appears to be confined to a channel.

The stratigraphical position of the Ninikaa Tuff has not been satisfactorily determined; however, it is very similar in composition to one sample of a tuff mapped as the Hasuma Tuff¹⁵. At least two different tuffs have been mapped as the Hasuma Tuff, and stratigraphical relations between these have not been determined. The particular sample similar to the Ninikaa Tuff lies ~25 m above the Tulu Bor Tuff in area 204.

Significance of the distribution of the Tulu Bor Tuff

Bowen¹² places the Suregei Complex about 80 m below the Tulu Bor Tuff in his section 130-1 along the western margin of the Suregei Cuesta. Findlater¹⁵ places the Allia Tuff 74 m below what Bowen and Vondra¹ identified as the Suregei Complex in areas 202 and 204; Acuff²⁸ finds a different thickness, but agrees on the stratigraphical order. We were therefore surprised to find that the Tulu Bor Tuff lies stratigraphically below the Allia Tuff in areas 204 and 207, and therefore must also underlie the so-called Suregei Complex of area 202. As the Suregei Complex lies below the Tulu Bor Tuff in the type area of both units, it is evident that the unit mapped as the Suregei Complex in area 202 must be a different stratigraphical horizon from that in area 15. Thus two different stratigraphical horizons have been correlated and mapped as the Suregei Complex. One of these lies near the base of the Plio-Pleistocene sequence in the Koobi Fora region; the other is much younger and lies nearer the middle of the sequence.

In order that these two units no longer be confused, we designate the white siltstones in area 102 as the Lokeridede

Complex, after the name of a small stream nearby. This unit lies below a major topographical break in slope and thus lies below the well exposed section in area 102. The nature of the contact is obscure, but the trend of the Lokeridede Complex differs from that of the overlying sediments, suggesting that it may lie below a slight angular unconformity. We accept the correlation of Williamson⁷ of this unit with the unit designated 'Suregei Tuff Complex' in area 202 because it seems to be situated in roughly the same position with respect to the KBS Tuff in area 110, and is lithologically similar. This complex is at about the level of the Burgi Tuff which is defined in the accompanying article. This unit is a very light grey montmorillonitic claystone containing cristobalite with parallel bedding. It is saline, with gypsum at the surface, and contains abundant recrystallized diatoms.

The base of the Suregei Complex is defined as the base of the Koobi Fora Formation. What has been erroneously identified as the Suregei Complex in area 202, however, overlies the Tulu Bor Tuff; furthermore, the Tulu Bor Tuff is widely exposed in what has been mapped as the Kubi Algi Formation, but in its type area belongs to the Koobi Fora Formation. Here a choice must be made; either the Koobi Fora Formation and the Kubi Algi Formation must be redefined, or the Kubi Algi Formation must be regarded as a very small part of the sedimentary sequence in the Koobi Fora region. At present we leave the definitions of the formations as they are, and apply those definitions throughout the Koobi Fora region—strata everywhere in the region which lie between the Suregei Complex and the Chari Tuff must be assigned to the Koobi Fora Formation.

Although we have not redefined the Koobi Fora Formation, certain strata formerly placed in the Kubi Algi Formation are now assigned to the Koobi Fora Formation. Figure 2 shows a composite section of the Koobi Fora Formation in which the old stratigraphical nomenclature is compared with the revised nomenclature. Stratigraphical terms in parentheses are formerly named units which were miscorrelated from their type localities. Stratigraphical thicknesses shown on the revised diagram are only our best estimates at present.

Discussion and conclusions

The most important implication of this work is that the type section of the Koobi Fora Formation includes neither of the marker horizons which were used to define the formation. The present type section begins at the Lokeridede Complex, not the Suregei Complex, and extends upward to levels above the Koobi Fora Tuff Complex, but not to the Chari Tuff. The

existing type section near the Koobi Fora spit requires correction, because the interval between the KBS Tuff and the Koobi Fora Tuff Complex (shown as ~80 m)¹⁴ is far too thin, and must be increased by nearly a factor of two^{2,29}. New sections which extend the present type section of the Koobi Fora Formation must be established which include the defining marker beds. It may be desirable to redefine the Koobi Fora Formation when such sections are established so that it includes the small sections below the Suregei Complex and above the Chari Tuff. As defined at present, the Kubi Algi Formation is only ~5 m thick, and is not lithologically distinct from the strata immediately above the Suregei Complex, thus there is no good reason for retaining that formation as a stratigraphical unit. Similarly, most of those strata presently assigned to the Guomde Formation should be included as part of the Koobi Fora Formation, because the two formations are not lithologically distinct². Strata belonging to the Guomde Formation as presently defined¹ have been mapped as part of the Upper Member of the Koobi Fora Formation in one area, and as part of the lower member of the Koobi Fora Formation in others^{12,15}.

The stratigraphical revisions proposed above are based solely on tuff correlation, but many of the same revisions, or variations on them, were proposed independently on the basis of palaeontological considerations^{4,6,7}. With the proposed revisions, most faunal correlation problems disappear without having to call on ecological differences to explain them. Fossil mammal assemblages in the Turkana basin appear, indeed, primarily to reflect age, as noted earlier³⁰; ecological effects seem subordinate.

Mapping, palaeomagnetic interpretations, definition of isotopic trends of carbonates and volcanic glass in time, and palaeogeographical reconstructions^{8,14,31} are all dependent on the lithostratigraphy, and earlier studies in these areas will have to be reconsidered (or amended). Although the revisions proposed above are important to the geological history of the area, the geographical positions of invertebrate and mammalian (and hominid) fossil specimens are not affected, and since they are recorded on aerial photographs³², their stratigraphical attributions can be checked. The Koobi Fora region is well known for its fossil hominids, but as most of these come from levels near or above the KBS Tuff, the present revisions will affect few of them.

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Table 1 Trace and minor element compositions of some tuffs in the Koobi Fora region and in the Shungura Formation

Area	Tuff	Sample	Fe ₂ O ₃	CaO	Ba	Mn	Nb	Ti	Y	Zn	Zr
Omo	Tuff A	F-133	3.70	0.21	109	824	153	1,285	140	253	1,419
117	Lokochot	75-117B	3.77	0.19	115	863	160	1,269	145	252	1,470
250	Lokochot	K80-295	3.58	0.19	123	837	152	1,263	141	239	1,403
251	Lokochot	77-30	3.62	0.18	114	809	152	1,201	135	250	1,407
260	Lokochot	K80-291	3.51	0.18	121	809	146	1,233	135	233	1,360
Omo	Tuff B- α	F-134	1.30	0.48	412	267	74	1,165	53	48	274
Omo	Tuff B- α	F-135	1.41	0.55	416	283	75	1,364	55	55	288
117	α -Tulu Bor	K80-183	1.51	0.65	491	350	70	1,533	49	58	380
129	α -Tulu Bor	77-24	1.25	0.61	427	240	72	1,437	52	53	280
129	α -Tulu Bor	77-27	1.46	0.61	452	326	70	1,452	50	55	353
204	α -Tulu Bor	K81-635*	1.26	0.48	407	250	77	1,185	53	56	272
260	α -Tulu Bor	K80-288	1.31	0.58	419	274	77	1,482	54	57	298
Omo	Tuff B- β	F-136	1.64	0.43	219	393	85	1,128	64	83	449
117	β -Tulu Bor	75-117c	1.55	0.40	152	364	82	940	61	74	349
117	β -Tulu Bor	K80-179	1.52	0.31	130	387	84	1,045	62	79	379
207	β -Tulu Bor	K81-460	1.45	0.33	143	372	81	925	61	87	351
204	Toroto	K80-307	4.65	0.15	286	932	78	1,626	98	209	905
207	Toroto	K80-279	4.68	0.21	296	1,017	86	1,705	104	211	947
204	Allia	77-31†	0.84	0.25	163	196	50	782	39	34	151
207	Allia	K81-694	0.84	0.38	168	188	52	775	39	37	156
Omo	C4	F-140	2.62	0.31	17	744	94	1,063	82	151	803
207	Ingumwai	K80-273	2.66	0.25	1	836	100	1,008	80	142	871
Omo	H2	F-101	3.22	0.22	27	832	201	1,467	131	236	1,306
Omo	H2	76-H2B‡	3.00	0.17	89	825	191	1,205	131	230	1,319
12	KBS	K80-134	3.12	0.18	7	887	204	1,215	137	253	1,276
15	KBS	K80-141	3.15	0.21	17	881	200	1,190	131	253	1,276
102	KBS	72-44§	3.07	0.19		850		1,080			
105	KBS	77-17	3.09	0.23	30	843	194	1,292	129	252	1,239
106S	KBS	K80-238	3.10	0.18	14	874	200	1,217	131	241	1,276
110	KBS	77-32¶	3.16	0.20	26	881	201	1,197	130	243	1,284
129	KBS	K80-222P#	3.25	0.38	30	891	196	1,564	127	228	1,314
130	KBS	K80-285	3.00	0.17	10	862	198	1,215	133	241	1,265
131	KBS	77-10	3.08	0.18	21	859	199	1,174	130	257	1,240
Omo	H4	F-105	4.78	0.38	70	1,907	143	3,578	83	204	1,007
112	Malbe	77-18**	4.81	0.43	120	1,921	139	3,360	84	196	990
112	Malbe	K80-225**	4.70	0.38	60	1,897	139	3,353	82	194	996
Omo	J7	228-11	2.46	1.12	740	989	80	2,731	60	95	661
119		K80-195	2.50	1.01	785	978	83	2,715	61	99	679
Omo	L	L‡	2.76	0.18	257	603	99	1,124	100	192	1,053
Omo	L	76-L‡	2.70	0.20	225	518	102	1,056	101	183	1,106
1	Chari	77-23	2.81	0.18	223	564	103	1,093	102	190	1,085
6A	Chari	K80-127	2.83	0.19	216	574	107	1,182	103	196	1,107
108	Chari	108-1‡	2.66	0.19	291	482	97	1,019	98	182	1,028
131	Chari	77-19	2.90	0.23	306	643	100	1,219	100	192	1,059
7	Silbo	7-106††	3.57	0.16	1	1,238	138	1,139	105	208	1,169
Kokoi	Silbo	K80-151	3.53	0.14	1	1,236	126	1,194	98	203	1,084
Shin	Silbo	K80-248**	3.52	0.20	18	1,219	131	1,264	99	200	1,101
Shin	Silbo	K80-251‡‡	3.66	0.17	5	1,231	134	1,281	99	203	1,109

* 15 m below Allia Tuff (77-31).

† Allia Tuff of Findlater^{7,9}.

‡ Pumice.

§ Analysis by electron microscope.

|| Previously mapped as Sargaei Tuff^{7,9}.¶ Previously mapped as Tulu Bor Tuff^{7,9}.

Composite pumice sample. Previously unmapped as any tuff.

** Previously mapped as KBS Tuff^{7,9}.†† Previously mapped as Chari Tuff^{7,9}.‡‡ Previously mapped as Okote Tuff^{7,9}.

Samples were prepared and analysed as described in ref. 15. New standards more similar in composition to our samples than the USGS rock standards have resulted in analytical results that are more accurate and more precise than those reported earlier¹⁶. Where statements are made concerning individual shards, the analyses were done by electron microprobe.

Results

Eight pairs of horizons in the two regions can be correlated on the basis of chemical compositions (Table 1). Tuffs A, B- α , B- β , C4, H2, H4, J7 and L from the Lower Omo Valley correspond to the Lokochot Tuff, the α -Tulu Bor Tuff, the β -Tulu Bor Tuff, the Ingumwai Tuff, the KBS Tuff, the Malbe Tuff, an unnamed tuff near the level of the Koobi Fora Tuff, and the Chari Tuff in the Koobi Fora region, respectively. These correlations together with definitions of new tuff names and complications are discussed below.

Tuff A-Lokochot Tuff: Tuff A is the lowest major tuff in the Shungura Formation, situated ~30 m above the lowest exposed sediments of the Shungura Formation. The Lokochot Tuff, in the Koobi Fora region, is named for a major ephemeral

stream channel, Il Lokochot, in the area east of Allia Bay; this tuff crops out extensively in this region and is also exposed in area 117, south of the Kokoi (see Fig. 1 for area designations and geographical names). The type exposure is located 200 m west of the point where the road crosses Il Lokochot. Here, the Lokochot Tuff is a 2.0-m thick bed of poorly stratified, grey, coarse to medium ash, with calcified rootcasts in its lower part. The glass shards are very light grey and clear or slightly frosted; they are stretched, bubblewall and platy shards. This tuff rests on a bed of greyish brown silty claystone with polyhedral fracture; irregular subvertical carbonate fillings occur in the upper part of the silty claystone. It is overlain by sandy siltstones and clayey siltstones. The strata in which it is located were formerly assigned to the Kubi Algi Formation⁵⁻¹⁴, although we believe that these beds should be assigned to the Koobi Fora Formation¹⁵. Analysis of glass separates (Table 1) from Tuff A and from the Lokochot Tuff are indistinguishable. This tuff is of interest because analyses of individual shards by electron microprobe fall into two distinct compositional groups (Fig. 2), both of which differ from the bulk composition of the glass separates; Tuff A also has this characteristic. It is possible

Table 2 Representative chemical analyses of some trace and minor elements from pure glass separates from marker tuffs at their type localities (see Table 3) in the Koobi Fora region

Tuff	Area	Sample	Fe ₂ O ₃	CaO	Ba	Mn	Nb	Rb	Ti	Y	Zn	Zr
Allia	204	77-31	0.84	0.37	163	196	50	129	782	39	34	151
Burgi	207	K80-274	3.56	0.22	28	1,357	211	157	1,548	104	192	1,254
Chari	1	77-23	2.81	0.18	223	564	103	102	1,093	102	190	1,085
Ileret Complex	6A	K80-144	4.91	0.23	5	1,732	239	162	1,993	131	318	1,653
	6	72-8	4.74	0.19	53	1,651	152	127	2,074	96	217	1,066
Ingumwai	207	K80-273	2.66	0.25	1	836	100	145	1,008	80	142	871
KBS	105	77-17	3.09	0.23	30	843	194	165	1,297	129	253	1,239
Koobi Fora Complex	101	K80-108	4.70	0.46	8	1,610	214	118	2,494	114	241	1,396
	101	K80-115	5.39	0.19	1	1,899	256	127	1,969	131	282	1,656
Lokochot	250	K80-295	3.58	0.19	123	837	152	132	1,263	141	239	1,403
Lorenyang	102	K80-104	2.20	0.15	1	1,448	56	152	900	84	153	823
Lower Ileret	6A	77-20	5.78	0.15	1	2,023	357	182	1,492	192	403	2,542
Malbe	112	K80-225	4.70	0.38	61	1,897	139	103	3,353	82	194	996
Moiti	260	K81-602	2.70	0.18	401	618	122	97	1,063	114	235	1,099
Ninikaa	116	75-116lt	4.05	0.39	285	1,889	129	85	3,725	72	173	784
	116	75-116dk	4.27	0.19	448	1,757	109	87	4,477	57	153	626
Okote Complex	131	77-9	4.76	0.20	34	1,595	215	149	1,847	124	256	1,493
	131	77-106B	3.81	0.72	1,048	1,183	137	79	1,470	102	184	1,179
Sigelib	—	K80-244	6.69	0.47	507	2,208	154	100	2,699	106	284	1,228
Silbo	—	K80-251	3.66	0.17	5	1,231	134	105	1,281	99	203	1,109
Toroto	207	K80-279	4.68	0.21	296	1,017	86	84	1,705	104	211	947
α -Tulu Bor	129	77-27	1.46	0.61	452	326	70	112	1,452	50	55	353
β -Tulu Bor	117	K80-179	1.56	0.32	135	387	83	112	930	63	79	366

Only one analysis is given for each tuff unless additional analyses of pure glass separates from the tuff showed compositional variability. Concentration is given in parts per million, except calcium and iron which are reported as the oxide in per cent. Total iron as Fe₂O₃.

that two tuffs erupted at different times may have very similar compositions, but the unique combination of finding two tuffs with identical bulk analyses and having two shard compositions mixed in the same ratio is very unlikely to have happened twice. Consequently, we regard the correlation of Tuff A and the Lokochot Tuff as one of the strongest ties between the Shungura Formation and the Plio-Pleistocene strata at Koobi Fora.

Tuff B-Tulu Bor Tuff: Tuff B of the Shungura Formation was divided into several units when it became clear that it represented several depositional events. We have analysed glass separates from tuffs from only the lowest two units, Tuff B- α and Tuff B- β . Although they differ slightly in composition they are chemically related (Table 1).

Several different stratigraphical horizons have been called the Tulu Bor Tuff⁵. Here, we use this name only for the tuff in area 129 (where it was originally named), and for those tuffs that have similar or indistinguishable compositions. The Tulu Bor Tuff in the Koobi Fora region has at least two variants; two of these correspond to Tuff B- α and Tuff B- β of the Shungura Formation (Table 1), and we refer to these as the α -Tulu Bor Tuff and β -Tulu Bor Tuff, respectively. Where a distinction is not necessary the term Tulu Bor Tuff is used. The type Tulu Bor Tuff in area 129 corresponds to Tuff B- α of the Shungura Formation (Table 1); this tuff is also found in areas 117, 204, 207 and 260. The tuff mapped as the Tulu Bor Tuff in area 117 (refs 8, 10) is in most places equivalent to Tuff B- β of the Shungura Formation (Table 1). However, in area 117 ~10% of the shards are compositionally similar to those of

the α -Tulu Bor Tuff, indicating that it has some older shards reworked into it.

In addition to the compositional similarity there are several other favourable points of comparison between Tulu Bor Tuff and Tuff B. Both are of normal polarity^{4,16}, and composed dominantly of flat bubble wall shards. The Tulu Bor Tuff lies ~35 m above the Lokochot Tuff, a distance comparable with the 40 m that separates Tuff B from Tuff A in the Shungura Formation. The intervening section of fluvial sediments is also similar.

Tuff C4-Ingumwai Tuff: Tuff C4 in the Shungura Formation lying ~110 m above Tuff B has normal polarity. Table 1 shows that sample K80-273 is chemically similar to Tuff C4. This is a sample of the lowest of four tuffs exposed in a cliff along Il Ingumwai (Fig. 1a). At this locality it lies ~50 m above the Tulu Bor Tuff. This tuff is here defined as the Ingumwai Tuff. It has a maximum thickness of ~0.5 m in this exposure, and is lenticular, pinching out to the west. It is pinkish grey to very pale yellowish grey, and consists of silt to medium sand sized glass shards. Most shards are light grey to greyish white; a few are very light brown. They are generally frosted. Most are stretched or frothy.

Tuff H2-KBS Tuff: Tuff H2 of the Shungura Formation has previously¹⁷ been correlated with the KBS Tuff in the Koobi Fora region on the basis of trace and minor element contents of glass separates from pumice, compositions of phenocrysts in pumice, and on the major and minor element content of glass shards analysed by the electron microprobe. We have since analysed glass separates from tuffs by X-ray fluorescence, and can now positively identify the KBS tuff in areas 10, 15, 130, 131, 105, 110 and south of area 106 (Table 1). Glass shards from the KBS Tuff are fairly distinctive. In general, the shards are clear: 60–90% are very light grey or colourless. The remainder grade from light grey to dark brown. This gives the tuff a 'salt-and-pepper' appearance in many localities. Occasionally the basal portion does not have the dark shards; Tuff H2 also lacks dark shards. The shards are a subequal mixture of bubble-wall stretched, platey and frothy types. Occasionally this tuff has <30% glass and should properly be called a tuffaceous sandstone (for example, area 102). Chemical analysis by electron microprobe indicates its presence in area 102 (ref. 17). The samples from area 110 and south of area 106 are from tuffs which had previously been mapped as the Tulu Bor Tuff and the Suregei Tuff, respectively^{7,9}; other tuffs mapped as the KBS Tuff can be shown to be different on the basis of their chemistries (see discussion below). Both Tuff H2 and the KBS Tuff lie in sections of normal polarity^{4,17}; the KBS Tuff has

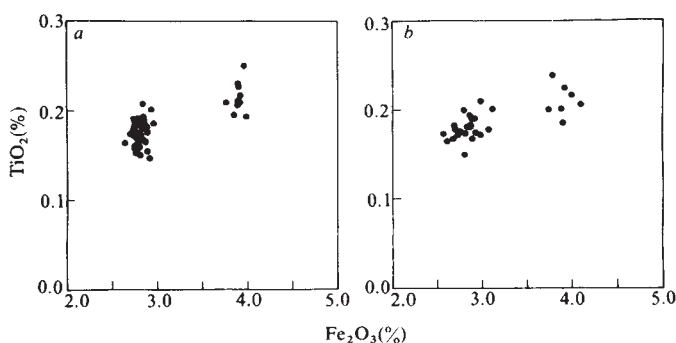


Fig. 2 Iron and titanium contents of individual glass shards from *a*, the Lokochot Tuff (59 shards) and *b*, from Tuff A (30 shards). Each point represents the average composition of an individual shard.

been dated at 1.89 ± 0.02 Myr (samples from areas 105 and 131 in ref. 19), a date comparable with that of Tuff H2 (1.88 Myr).

Tuff H4-Malbe Tuff: Tuff H4 occurs 15 m above Tuff H2 in the Shungura Formation. Tuff H4 has previously¹⁷ been correlated with a tuff in the Koobi Fora region, which we now designate as the Malbe Tuff, named for Kolom Malbe, along which this tuff is well exposed. This tuff occurs ~15 m above the KBS Tuff in the eastern portion of area 105. Analyses of glass separates from this tuff are given in Table 1. This tuff has pumice clasts of variable composition, which are not included in Table 1, but which are discussed elsewhere^{17,18}. Glass separates from these pumices fall on a single compositional trend, and may represent eruptive products of different portions of a compositionally variable magma chamber¹⁸. Electron microprobe studies of individual shards show that the entire range of glass compositions spanned by the pumice glasses is present in the tuff¹⁸. Although McDougall *et al.*¹⁹ do not distinguish this tuff from the KBS Tuff, samples of this tuff give a K/Ar age of 1.88 ± 0.02 Myr (samples from area 112 of ref. 19).

In its type area the Malbe Tuff is confined to a channel, and reaches a maximum thickness of ~2 m. It can be distinguished from the KBS Tuff in the field wherever it contains pumice, because the pumice clasts of the Malbe tuff are an assemblage of very dark brown (nearly black), medium grey and white types, whereas those of the KBS Tuff are dominantly grey or white, with very rare (<1%) dark pumice.

Tuff J7-Koobi Fora Tuff (*sensu lato*): Tuff J7 of the Shungura Formation occurs in sediments of reversed polarity. An unnamed tuff in area 119 (K80-195) has essentially the same composition as Tuff J7 (Table 1). This tuff is at about the same level as the Koobi Fora Tuff, which also has reversed polarity¹⁶. This sample is ~5 m below a tuff which is compositionally the same as a sample from the Koobi Fora Tuff in area 101.

The Ileret Tuff Complex, the Okote Tuff Complex and the Koobi Fora Complex, or variations on these names, are found near Ileret, along the Karari Ridge and near Koobi Fora spit, respectively. These tuffs have been correlated^{9,10} because they are tuffaceous intervals above the KBS Tuff. We have analysed over 30 glass separates from pumice and tuffs from these localities and have found only one compositionally distinct set of samples. They are found at the base of the Ileret Tuff

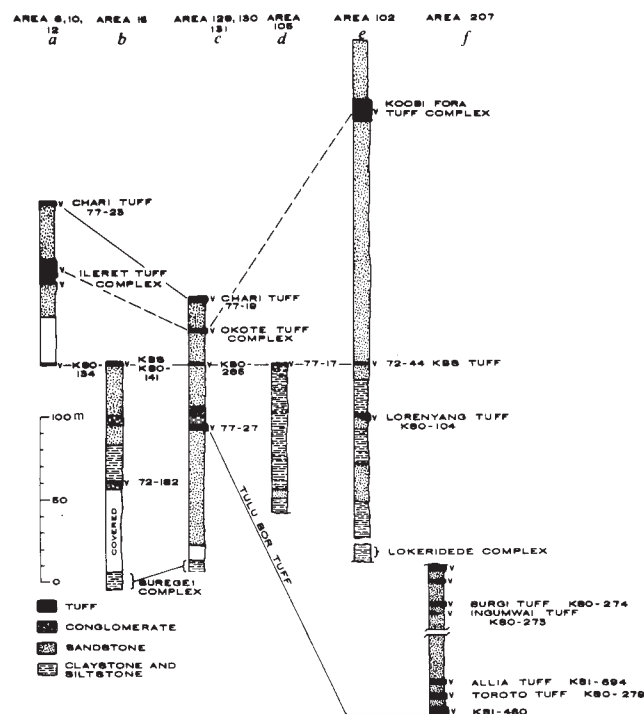


Fig. 3 Stratigraphical sections of Plio-Pleistocene sediments in the Koobi Fora region. Section a by Bowen⁵ and T.E.C.; section b by Bowen⁵; section c by Bowen⁵ and T.E.C.; section d by T.E.C.; section e by T.E.C.; section f by F.H.B. and T.E.C.

Complex. Thus, we designate this tuff as a separate unit, the Lower Ileret Tuff. This tuff is medium to dark grey, has fine to medium shards, and is ~1 m thick. The shards are dark grey and are dominantly frothy. It has an abrupt basal contact and overlies a brown mudstone with prismatic structure. It is overlain by 3–4 m of interbedded sandstones and siltstones, which give way to the tuffaceous sand and silts of the Ileret Tuff Complex.

The Ileret, Koobi Fora and Okote Tuff Complexes have variable chemistries as shown by major, minor and trace element analyses of glass separates from pumice and from tuff.

Table 3 Type localities of marker horizons in the Koobi Fora region

Marker horizon	Type area	Sample	Locality* Photo	X	Y	Bowen and Vondra ⁶	White and Harris ²⁵ Harris ²⁶	Findlater ⁸	Findlater ¹⁰	Findlater ⁹
1 Allia Tuff	204	77-31	1,574	167-056	—	—	Allia	4.5	Allia	Allia
2 Burgi Tuff	207	K80-274	1,729	083-093	—	—	—	—	—	—
3 Chari Tuff	1	77-23	1,467	077-122	—	Chari	Chari	Chari	Chari	Karari
4 Ileret Tuff Complex	6A	K80-144	1,398	135-145	—	—	Lower/Middle	Middle	Okote	Okote
5 Ingumwai Tuff	207	K80-273	1,729	083-093	—	—	—	—	—	—
6 KBS Tuff	105	77-17	1,591	142-109	—	KBS	KBS	KBS	KBS	KBS
7 Koobi Fora Tuff Complex	101	K80-108	1,343	103-174	—	—	Koobi Fora	BBS	Okote	Okote
8 Lokeridede Complex†	102	K80-126	1,414	120-168	—	Suregei	102/T.II	Surgaei	Suregei	Surgaei
9 Lokochot Tuff	250	K80-295	1,550	042-138	—	—	250/T.I	—	—	—
10 Lorenyang Tuff	102	K80-104	1,413	150-022	—	Tulu Bor	102/T.IV	Tulu Bor	Tulu Bor	Tulu Bor
11 Lower Ileret Tuff	6A	77-20	1,397	127-043	—	—	Lower/Middle	Lower†	Okote	Okote
12 Malbe Tuff	112	K80-225	1,656	040-102	—	—	—	—	—	KBS
13 Moiti Tuff	260	K81-602	1,435	143-096	—	—	—	—	—	—
14 Ninikaa Tuff	116	75-1,161	1,589	057-115	—	—	116/T.II	Tulu Bor	Tulu Bor	Tulu Bor
15 Okote Tuff Complex	131	77-9	1,750	049-124	—	—	Okote	BBS	Okote	Okote
16 Sigelib Tuff	—	K80-244	1,815	035-152	—	—	—	—	—	KBS
17 Silbo Tuff	—	K80-251	1,741	155-072	—	—	—	—	—	Okote
18 Suregei Complex†	15	K80-138	1,600	074-033	—	Suregei	Suregei	Surgaei	Suregei	Surgaei
19 Toroto Tuff	207	K80-279	1,729	035-160	—	—	—	—	—	—
20 α-Tulu Bor Tuff	129	77-27	1,648	055-114	—	Tulu Bor	Tulu Bor	Tulu Bor	Tulu Bor	Tulu Bor
21 β-Tulu Bor Tuff	117	K80-179	1,456	086-152	—	—	117/T.III	Tulu Bor	Tulu Bor	Tulu Bor

Representative compositions of tuffs from the type locality are given in Table 2. Other names that have been used for these horizons in their type localities are also included. The type localities are shown in Fig. 1.

* Localities are given by coordinates of type locality on 9×9-inch photographic prints of area taken in December 1970 by Hunting Surveys, London. X and Y coordinates are in millimetres from the left edge and from the top edge of print, respectively.

† Contains diatoms. Probably recrystallized diatomite.

‡ Captions for Figs 3 and 5 in ref. 8 are transposed.

also analysed numerous tuffs in the Shungura Formation, although we have done less than one-third of the total. There are now eight positive ties between the Koobi Fora region and the Lower Omo valley and more correlations are expected with analysis of additional samples from the Shungura Formation. Thus, many tuffs occur in both sequences, and approximately the same number of tuffs occur in any given interval in both sequences, contrary to earlier belief²².

If stratigraphical sections from different localities in the Koobi Fora region are examined in detail, several significant features are noted (Fig. 3). In area 129, some 30 m of section separates the KBS Tuff (1.89 Myr in age¹⁹) and the Tulu Bor Tuff (>3 Myr age⁴). In area 102, there is >100 m of lacustrine section below the KBS Tuff; the Tulu Bor Tuff is not found in area 102. This lacustrine sequence (the laminated siltstone facies of Bowen^{5,11}) is about the same age and thickness as the lacustrine interval from G14 to G28 of the Shungura Formation, and so should be considered as broadly correlative, as suggested by Brown *et al.*²³. However, in area 207, south of Kubi Algi, ~70 m of section occurs above the Tulu Bor Tuff; these sediments are not similar to the laminated siltstones in area 102, but rather represent fining-upward fluvial cycles, and are in fact equivalent to members B and C of the Shungura Formation which is also characterized by fining-upward cycles. This suggests that a considerable thickness of sediment was deposited in the Koobi Fora region between Tulu Bor and KBS times.

However, in some areas (such as area 129) almost the entire thickness was removed before deposition of the KBS Tuff. In area 129, we believe that erosion exposed the Tulu Bor Tuff and that the present dip slope of the Tulu Bor Tuff may represent an exhumed land surface. In area 15, erosion probably removed the Tulu Bor tuff entirely. Based on correlation with the Shungura Formation, this erosion must have taken place between the time represented by member D and that represented lower part of member G, or between ~2.4 and ~2 Myr ago.

We can now account for much of the sediment thought to be missing in the Koobi Fora region²³. Stratigraphical thicknesses between marker beds in the Shungura Formation and the Koobi Fora Formation are often quite similar (Fig. 4). This, combined with the presence of tuffs in both the Koobi Fora region and the Lower Omo Valley, implies that the two sequences were probably part of the same depositional system. Differences in thicknesses can sometimes be attributed to erosion, as well as to primary differences in thicknesses.

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Eukaryotic ribosomes can recognize preproinsulin initiation codons irrespective of their position relative to the 5' end of mRNA

Peter T. Lomedico & Stephen J. McAndrew

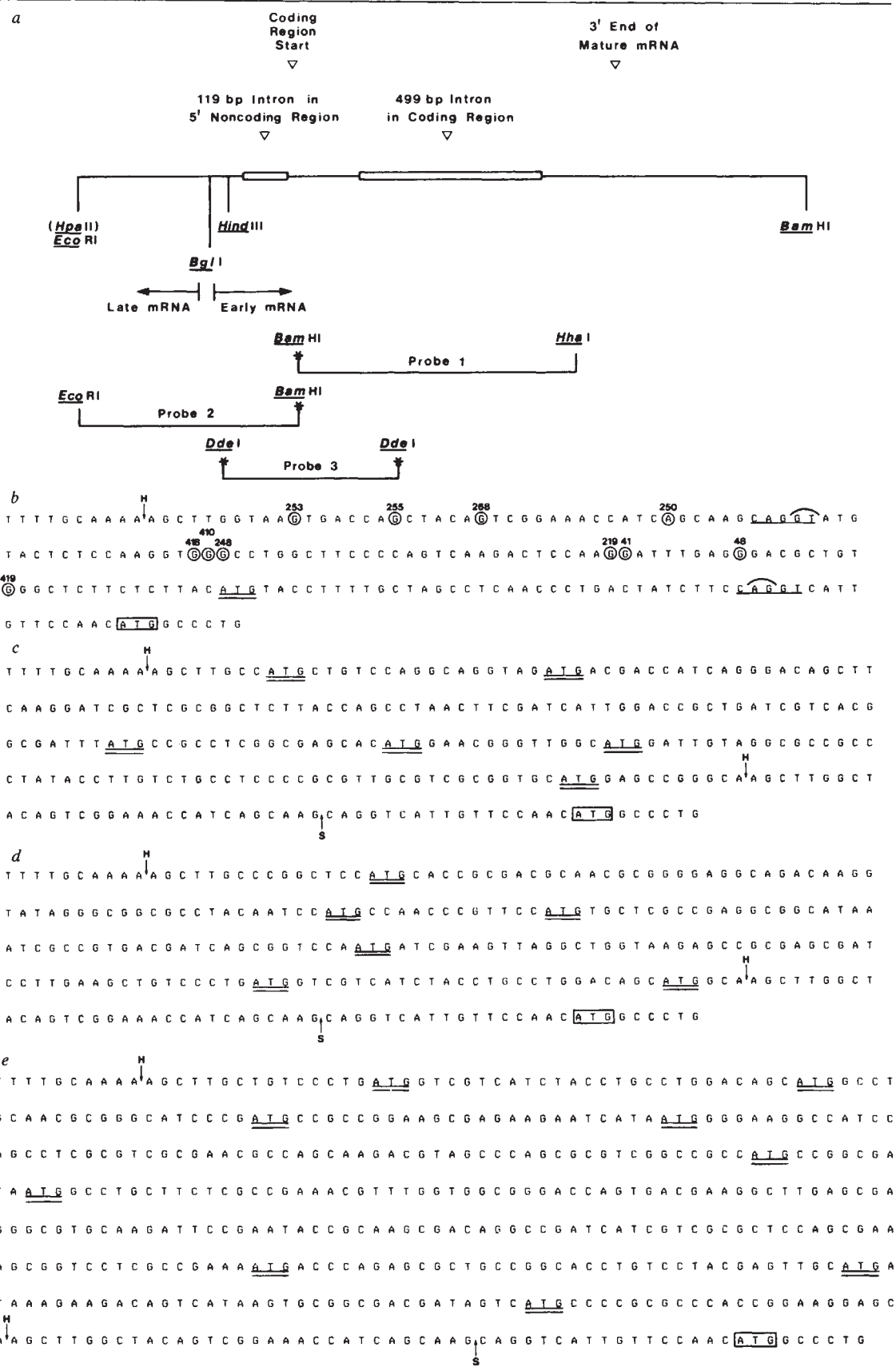
Department of Molecular Genetics, Hoffmann-La Roche Inc., Nutley, New Jersey 07110, USA

The functional assay of a eukaryotic mRNA, into which additional AUG codons have been introduced by in vitro mutagenesis, shows that a translational initiation site need not necessarily be the nearest AUG codon to the 5' end of a mRNA. Hence, the sequence surrounding the AUG, and not simply its position relative to the 5' end of mRNA, appears to be important in determining initiation efficiency.

How does a ribosome decide where to begin translation on a eukaryotic messenger RNA? In prokaryotic mRNAs, initiation codons are preceded by a conserved, purine-rich sequence which plays some part in directing the ribosome to the correct AUG codon. In contrast, comparison of 5'-noncoding regions

of eukaryotic mRNAs fails to reveal any sequence homology or conservation, casting doubts on the existence of a sequence functionally analogous to the bacterial ribosome binding site¹. One simple hypothesis to explain initiation codon selection by the eukaryotic ribosome is that translation begins at the first

Fig. 1 *a*, The construction of prI2/SV40-46 has been described previously³. Briefly, a *Hind*III site was engineered into the 5'-noncoding region of the cloned rat insulin II gene¹⁸, upstream of the 119 bp intron that interrupts this region. A 416 bp *Hpa*II-*Hind*III fragment from SV40 (previously cloned¹⁹ in a fashion that converted the *Hpa*II site to an *Eco*RI site) was ligated to the *Hind*III site in the insulin gene, and the resultant hybrid gene was cloned between the *Eco*RI and *Bam*HI sites of pBR322. *b*, The sequence across the *Hind*III site (H) of prI2/SV40-46 shows that the SV40 sequence is in the correct orientation connected to the 5'-noncoding region of the insulin II gene. The nucleotide redundancies at the junctions of the 119-bp intron are underlined, while the GT and AG dinucleotides at the beginning and end of this intron are overlined (the nucleotide sequence across this splice site in mature insulin II mRNA¹⁸ is AAGCAG-GUCAU, 5'→3'). The preproinsulin initiation codon is boxed and an ATG triplet in the intron is doubly underlined. Deletion mutants were created by digesting prI2/SV40-46 with *Hind*III, treating with exonuclease *Bal*31²⁰ and attaching *Hind*III linkers (Collaborative Research, Catalog no. 18067, CAAGCTTG); following digestion with *Hind*III, the deleted gene fragments were purified by polyacrylamide gel electrophoresis and cloned (via the *Hind*III site) next to the SV40 early region promoter described above. Hence, each deletion extends from the *Hind*III site of prI2/SV40-46 into the insulin gene 5'-noncoding region, with no alteration to the SV40 sequences. The cloned deletion mutants were characterized by sequence analysis. The end point of each deletion is circled and numbered (for example, the sequence across the *Hind*III site of mutant Δ255 and *Alu*I (403 bp) is TGCAAAAAGCTTGGCTACA, 5'→3'). *c*, *d* and *e*, Insertion mutants were created by attaching *Hind*III linkers to *Hae*III (213 bp) fragments of pBR322, and inserting these into the *Hind*III site of Δ255. Individual clones were characterized by restriction analysis and the inserts were completely sequenced. The *Hae*III fragment was cloned in both orientations in ΔprI2/SVO 255J2 (*c*) and ΔprI2/SVO 255J5 (*d*) and the *A*1uI fragment in one orientation, ΔprI2/SVO 255L3 (*e*). Each sequence shows both *Hind*III sites (H), the preproinsulin initiation codon (boxed ATG), and the ATG triplets in the insert (doubly underlined); for clarity, the 5'-noncoding region intron is deleted (s denotes where this intron resides in the plasmid DNA), hence, substituting U for T, this is the predicted sequence of the spliced mature mRNA. For convenience, these plasmids will be abbreviated as Δ255J2, Δ255J5 and Δ255L3.



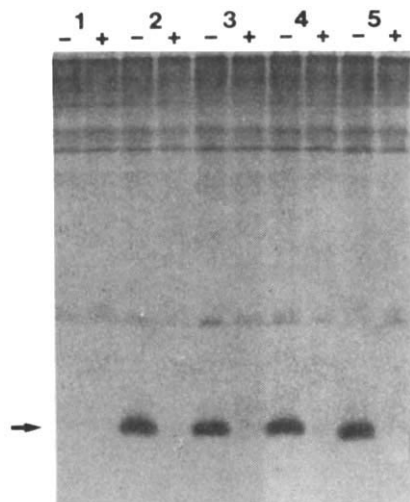


Fig. 2 Calcium phosphate DNA precipitate suspensions were formed²¹ using plasmid DNA at $20 \mu\text{g ml}^{-1}$ and presented to COS cells as described by Chu and Sharp²². One-fifth of the cells were plated in a 6-cm dish and the remainder in a 15-cm dish. Following a 5-h incubation at 37°C in 5% CO_2 , the DNA supernatant was removed and the cells were shocked with 25% glycerol in media for 1 min²², rinsed and returned to the incubator with fresh media. After 3 days, RNA was isolated from the 15-cm dish, and the cells in the 6-cm dish were labelled with ^{35}S -cysteine²³ for 4 h and solubilized with detergents²⁴. Equal amounts of solubilized proteins were reacted with antiserum to bovine insulin (Miles) in the presence (+) and absence (-) of an excess of bovine insulin (Sigma; $10 \mu\text{g}$) as competitor²⁵. The antigen-antibody complexes were recovered²⁶ using *Staphylococcus aureus* bacteria (Pansorbin; Calbiochem), extracted with 8 M urea/1% SDS/0.1 M dithiothreitol at 100°C and electrophoresed on 15% acrylamide/SDS gels²⁷. The acrylamide gel was impregnated with fluor (Enhance, NEN), dried down and exposed to X-ray film at -80°C . 1, pSVO; 2, prI2/SV40-46; 3, $\Delta 250$; 4, $\Delta 255$; 5, $\Delta 268$. Plasmid pSVO is a pBR322 derivative which contains only the 416-bp SV40 origin of replication. Insulin antigen released into the culture media from transfected cells was measured by radioimmunoassay (Amersham; this assay uses human insulin as a standard and expresses concentrations in $\mu\text{U ml}^{-1}$ where pure insulin is ~ 25 units per mg): 23, 270, 206, 214 and $144 \mu\text{U ml}^{-1}$ were found in cultures 1-5, respectively.

AUG codon at the 5' end of the mRNA. As embodied in the scanning ribosome model², this hypothesis explains many characteristics of initiation by eukaryotic ribosomes (for example, the absence of polycistronic eukaryotic mRNAs). The 'first AUG' hypothesis predicts that there is no eukaryotic equivalent of a bacterial ribosome binding site, and that the 5'-proximal AUG triplet alone, with no influence from surrounding sequences, signals where polypeptide chain synthesis will initiate. We have directly tested this hypothesis by analysing mutations introduced in the 5'-noncoding region of the rat insulin II gene.

Experimental strategy

For a particular eukaryotic mRNA, if an AUG triplet is introduced into the 5'-noncoding region (upstream of the normally used initiation codon), the simple interpretation of the 'first AUG' hypothesis predicts: (1) if this new AUG codon is in the normal reading frame with no intervening termination codons, then translation will result in the expected polypeptide with an N-terminal extension; or (2) if this new AUG codon is out of the normal reading frame, then translation will not generate the expected protein. To test these predictions, we have used a rapid expression assay to evaluate rat (pre)proinsulin synthesis programmed by mutants of plasmid prI2/SV40-46³. This recombinant plasmid (see Fig. 1a) was constructed by connecting the 5'-noncoding region of the SV40 large T/small T antigen gene to the 5'-noncoding region of the rat insulin II gene. We

have previously shown that when introduced into COS cells (simian cells transformed by origin-defective SV40 mutants)⁴, the SV40 early region promoter in this construct functions to drive transcription yielding a correctly spliced hybrid SV40-insulin mRNA in which the 5'-proximal AUG triplet is the normally used rat preproinsulin initiation codon. This mRNA is translated to generate preproinsulin, which is rapidly processed to proinsulin. This system permits the rapid analysis of cloned, *in vitro* engineered mutations necessary to test the 'first AUG' hypothesis.

Construction of deletion mutants

Plasmid prI2/SV40-46 carries a unique *Hind*III site, which facilitates the engineering of AUG codons into the 5'-noncoding region of the hybrid SV40-insulin mRNA. Unfortunately, downstream from this *Hind*III site is a termination codon that is in the preproinsulin reading frame. To correct this, deletion mutants were generated using exonuclease *Bal*31 (Fig. 1b). Mutants $\Delta 255$, $\Delta 268$ and $\Delta 250$ all carry small deletions (11, 17 and 30 base pairs (bp), respectively) in the insulin 5'-noncoding region, which remove this in-frame termination codon. In COS cells, these mutants, and the parental plasmid, all programme (pre)proinsulin synthesis to similar levels (Fig. 2), demonstrating that the deleted nucleotides have no obligatory role in initiation codon selection.

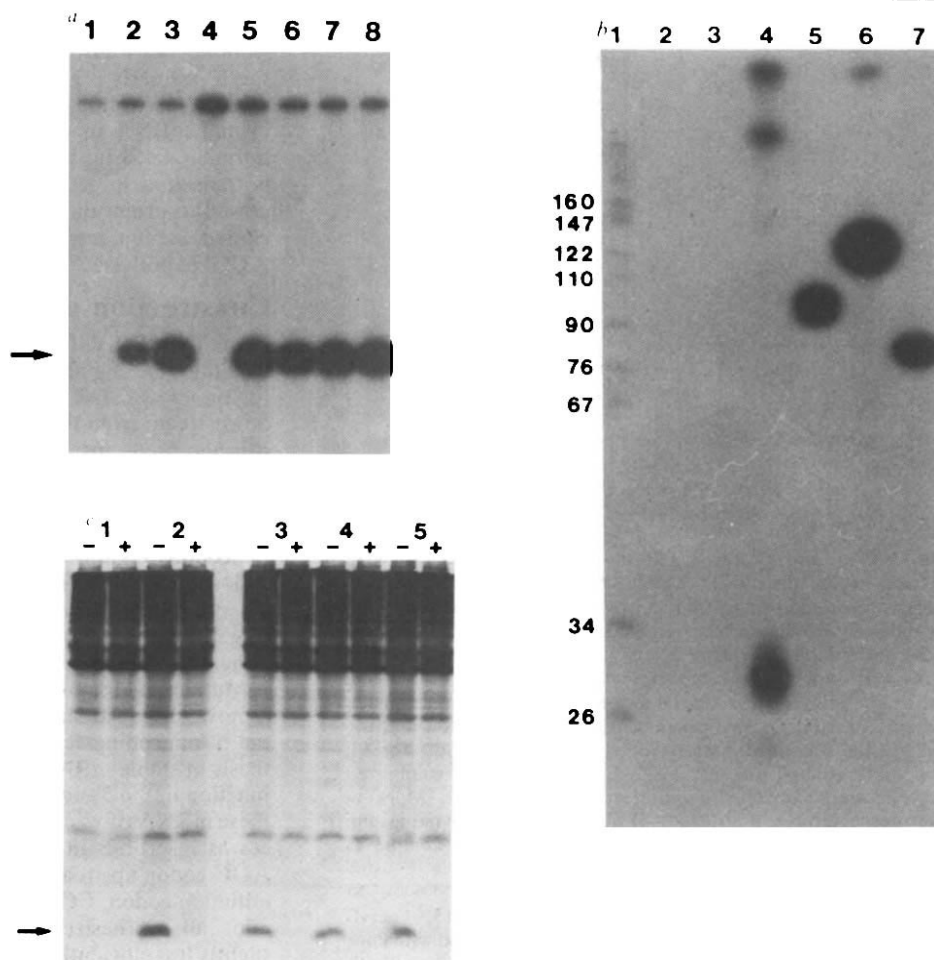
Mutants $\Delta 418$, $\Delta 41$ and $\Delta 419$ carry larger deletions, which remove the upstream splice junction from the intron lying in the 5'-noncoding region. These mutants programme the synthesis of stable mRNA (Fig. 3a), in which the remaining splice junction in the 5'-noncoding region is not used (Fig. 3b). Thus, these mRNAs now carry the previously excised intronic sequences as insertions in the 5'-noncoding region, creating a new AUG codon upstream, but out of frame with the normally used initiation codon. COS cells transfected with these mutant plasmids all synthesize and secrete proinsulin (Fig. 3c), albeit slightly less efficiently than the parental plasmid. Hence, in spite of the upstream AUG, ribosomes still recognize the normally used initiation codon, clearly violating a prediction of the 'first AUG' hypothesis.

Construction of insertion mutants

To create mutants with upstream, in-frame AUG codons, fragments of pBR322 were cloned into the *Hind*III site of $\Delta 255$. Mutants $\Delta 255\text{J5}$ and $\Delta 255\text{J2}$ contain a 213-bp *Hae*III fragment, carrying six AUG codons, cloned in both orientations (Fig. 1c and 1d). Mutant $\Delta 255\text{L3}$ contains a 403-bp *Alu* fragment, carrying nine AUG codons, inserted in one orientation (Fig. 1e). The mRNAs produced by $\Delta 255\text{J5}$ and $\Delta 255\text{L3}$ will have the 5'-proximal AUG in the insulin reading frame with no intervening termination codons; all other AUG triplets are out of frame. For the mRNA produced by $\Delta 255\text{J2}$, only the sixth AUG triplet from the 5' end will be in the insulin reading frame. These mutants programme the synthesis of stable, correctly spliced mRNA (Fig. 4a) which contain the inserted pBR322 sequences intact (Fig. 4b). COS cells transfected with these mutant plasmids all synthesize proinsulin, but much less efficiently than the parental plasmid (Fig. 4c). A large insulin-immunoreactive protein (molecular weight (M_r) $\sim 21,000$) is seen in $\Delta 255\text{J5}$ transfected cells (Fig. 4c). Pulse-chase experiments (Fig. 4d) indicate that this larger protein is somewhat unstable (because of incorrect processing?) and does not chase into proinsulin.

These experiments indicate that ribosomes can recognize the normally used insulin initiation codon, in spite of it being the 7th (in $\Delta 255\text{J2}$ and $\Delta 255\text{J5}$) or 10th (in $\Delta 255\text{L3}$) AUG from the 5' end. This conclusion is supported by the ability to generate preproinsulin in a cell-free translation using mRNA from all three transfected cells (data not shown). Apparently, the first AUG is also being used on the $\Delta 255\text{J5}$ mRNA, leading to the synthesis of an $\sim 21,000$ - M_r , which probably consists of preproinsulin (110 amino acids) with an N-terminal extension (87 amino acids). Thus, there are two functional initiation codons

Fig. 3 COS cells were transfected with different plasmids as described in Fig. 2. RNA was prepared from the 15-cm dish and examined by S_1 mapping²⁸ using end-labelled probes²⁹. *a*, Probe 1 (see Fig. 1*a*) is a 750-bp fragment uniquely 3'-end-labelled at a *Bam* site in the coding region, extending to a *Hha* site on the other side of the 499-bp intron in the coding region. If a correctly spliced mRNA anneals to the labelled anti-sense DNA strand, nuclease S_1 will digest the unhybridized intronic sequences, leaving protected a 170-nucleotide labelled fragment (designated by the arrow on the photograph of the X-ray film). 1, No RNA; 2, 10 ng of rat insulinoma poly(A⁺)RNA³; 3, 50 ng of rat insulinoma poly(A⁺)RNA; 4–8, equal amounts of RNA (1/10th of the sample) from cells transfected with pSVO, prI2/SV40-46, $\Delta 41$, $\Delta 418$, and $\Delta 419$, respectively. There are $\sim 5 \times 10^7$ molecules of preproinsulin II mRNA in 10 ng of rat insulinoma poly(A⁺)RNA³. *b*, Probe 2 (see Fig. 1*a*) is a 600-bp fragment from prI2/SV40-46 uniquely 5'-end-labelled at a *Bam* site in the coding region, extending upstream to the SV40 *EcoRI* site. If a correctly spliced mRNA anneals to the labelled anti-sense DNA strand, nuclease S_1 will digest the unhybridized intronic sequences, leaving protected a 29-nucleotide labelled fragment. In the deletion mutants, if the intronic sequences are not spliced out, then S_1 will digest at the deletion end point. 1, 32 P-pBR322/*Hpa*II molecular weight numbers (chain length in nucleotides shown alongside); 2, no RNA; 3–7, equal amounts of RNA (1/10th of the sample) from cells transfected with pSVO, prI2/SV40-46, $\Delta 41$, $\Delta 418$ and $\Delta 419$, respectively. *c*, Transfected cells (6 cm dish) were labelled with 35 S-cysteine and the solubilized proteins processed as described in Fig. 2. 1–5, pSVO, prI2/SV40-46, $\Delta 41$, $\Delta 418$ and $\Delta 419$, respectively. Radioimmunoassay revealed 27, 288, 95, 83 and 124 μ U ml⁻¹ of insulin antigen in the spent media from cultures 1–5, respectively.



on the $\Delta 255J5$ mRNA; the initiation codon closer to the 5'-terminus appears to be used more frequently (Fig. 4*d*) even though it probably does not function in *E. coli*⁵. No larger proteins are detected in $\Delta 255J2$ and $\Delta 255L3$ transfected cells, implying that these upstream in-frame AUG triplets are not used as initiation codons. It is possible that these undetected proteins might be rapidly degraded, incapable of reacting with insulin antibodies, or rapidly processed to proinsulin, but we consider this unlikely based on the properties of the 21,000-*M_r* protein programmed by $\Delta 255J5$, the immunological characteristics of preproinsulin⁶, and the cell-free translation results which confirm the *in vivo* findings.

Implications for initiation codon selection

These data demonstrate that eukaryotic ribosomes are capable of initiating polypeptide chain synthesis at internal AUG codons, that multiple initiation sites can be used on the same mRNA, that some AUG triplets never function as initiation codons and that proximity to the 5' end modulates the efficiency of initiation codon utilization. The mere presence of an AUG triplet at the beginning of a mRNA, as predicted by the 'first AUG' hypothesis, is simply not enough information to signal translation initiation. It is likely that the structure surrounding an AUG triplet, as well as its location, are important for efficient initiation. Possibly the system described here could be used to study systematically the sequence requirements involved in initiation codon selection. Also, more experiments are necessary to see if inefficient initiation at internal AUG codons

is the result of the presence of multiple upstream AUG triplets, the increased distance between the internal initiation codon and the 5' end of the mRNA, or an altered structure around the AUG codon.

Other work lends support to the notion that the 'first AUG' hypothesis is not tenable, and that initiation codon proximity to the 5'-terminus has some role in translational efficiency. There are several eukaryotic mRNAs in which translation appears to initiate at an internal AUG triplet⁷⁻¹⁰ or which appear to possess two functional initiation codons^{11,12}. Mulligan and Berg¹³ have shown using a SV40 vector system that an internal AUG codon is used in bacterial xanthine-guanine phosphoribosyl transferase synthesis in mammalian cells; deletion of the upstream AUG triplets results in more efficient translation. Rosenberg and Paterson have demonstrated that the 5'-proximal coding sequence in a polycistronic prokaryotic mRNA can be translated in a eukaryotic cell-free system if this RNA carries a 5'-terminal cap structure^{14,15}; genes encoded by internal cistrons can be expressed if they are moved closer to the 5'-terminus^{15,16}. Finally, Young *et al.*¹⁷ have suggested that the different lengths of the 5'-noncoding region of mouse α -amylase mRNA may be responsible for differential amylase expression in liver and salivary gland.

If ribosomes can initiate at internal AUG codons, why are there no polycistronic mRNAs and why is the first AUG triplet used as an initiation codon on the vast majority of mRNAs? Kozak, in a modified scanning model^{7,8}, suggests that a 40S ribosomal subunit moves along the mRNA, starting from the

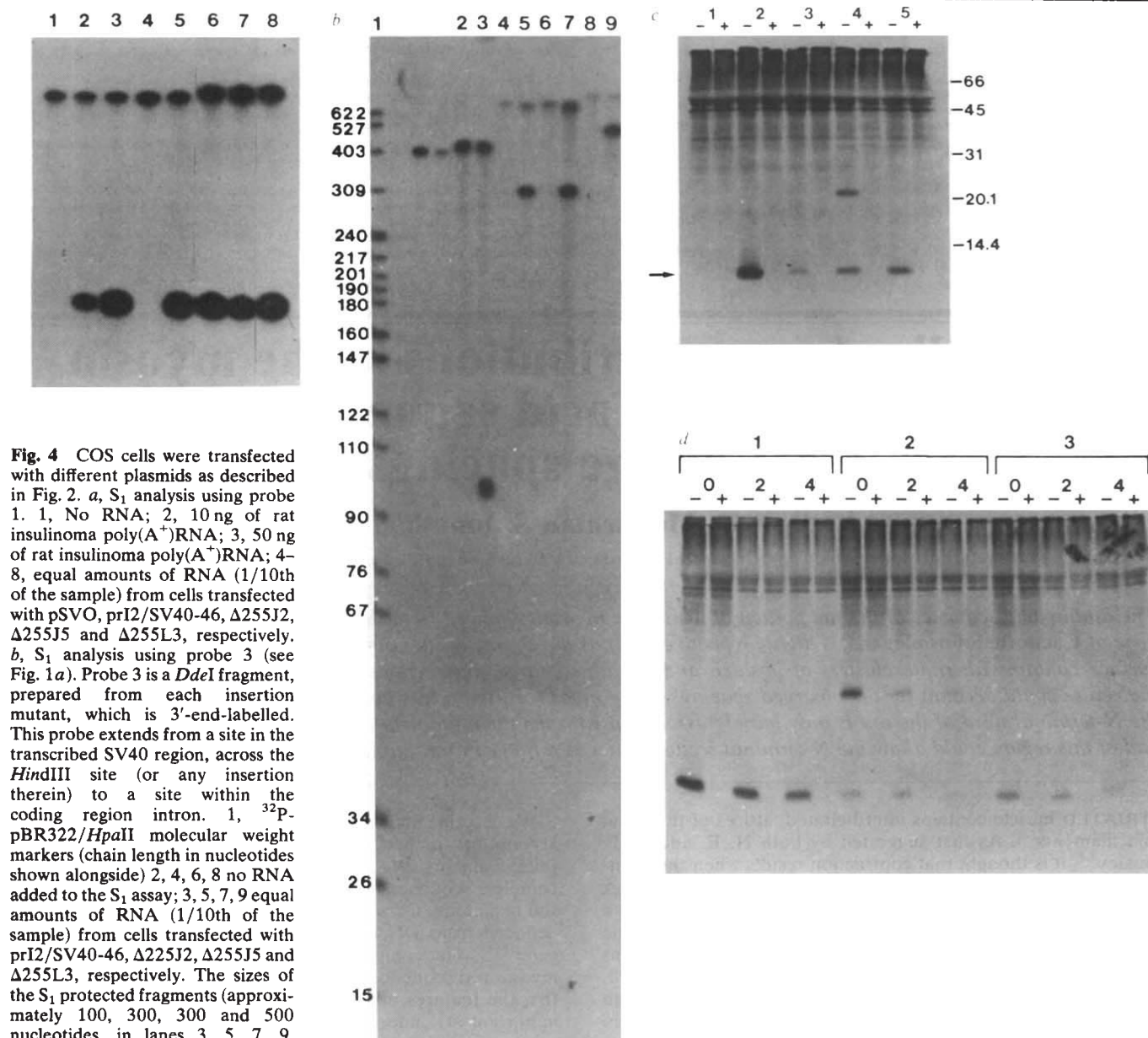


Fig. 4 COS cells were transfected with different plasmids as described in Fig. 2. *a*, S₁ analysis using probe 1. 1, No RNA; 2, 10 ng of rat insulinoma poly(A⁺) RNA; 3, 50 ng of rat insulinoma poly(A⁺) RNA; 4-8, equal amounts of RNA (1/10th of the sample) from cells transfected with pSVO, prI2/SV40-46, Δ255J2, Δ255J5 and Δ255L3, respectively. *b*, S₁ analysis using probe 3 (see Fig. 1*a*). Probe 3 is a *Dde*I fragment, prepared from each insertion mutant, which is 3'-end-labelled. This probe extends from a site in the transcribed SV40 region, across the *Hind*III site (or any insertion therein) to a site within the coding region intron. 1, ³²P-pBR322/*Hpa*II molecular weight markers (chain length in nucleotides shown alongside); 2, 4, 6, 8 no RNA added to the S₁ assay; 3, 5, 7, 9 equal amounts of RNA (1/10th of the sample) from cells transfected with prI2/SV40-46, Δ225J2, Δ255J5 and Δ255L3, respectively. The sizes of the S₁ protected fragments (approximately 100, 300, 300 and 500 nucleotides, in lanes 3, 5, 7, 9, respectively) indicate that S₁ is attacking at the 5'-noncoding region upstream splice junction, and that the inserted sequences are preserved intact within these mRNA. Analysis using probe 2 confirms that the 5'-noncoding region intron is spliced normally in all these mRNAs. *c*, Transfected cells (6 cm dish) were labelled with ³⁵S-cysteine and the solubilized proteins processed as described in Fig. 2. 1-5, pSVO, prI2/SV40-46, Δ255J2, Δ255J5 and Δ255L3, respectively. Molecular weight markers (weight in daltons × 10⁻³) were run in a parallel lane and visualized by Coomassie blue staining. Radioimmunoassay revealed 22, 117, 29, 89 and 42 μU ml⁻¹ of insulin antigen in the spent media from cultures 1-5, respectively. *d*, Transfected cells were labelled with ³⁵S-cysteine for 15 min and collected immediately (0 h), or subjected to a 2 or 4 h chase (the radioactive media were removed and the monolayer was rinsed several times with complete media). The solubilized proteins were processed as described in Fig. 2. 1, prI2/SV40-46; 2, Δ255J5; 3, Δ255L3.

5' end, searching for AUG codons. The model predicts that flanking sequences modulate the recognition of functional AUG initiation codons by the migrating ribosomal subunit; if synthesis is not initiated at a particular AUG triplet, the scanning process continues until a functional initiation site is encountered. Functional initiation codons are found most frequently in the sequence $\hat{C}XXAUGG$. Kozak proposes that this process works most efficiently when the first AUG triplet is the functional initiation codon, thus there is a strong selective pressure against mutations that create new AUG triplets in the 5'-noncoding region. Furthermore, nature may use this process as a translational control mechanism: by placing a particular polypeptide's synthesis under the control of an internal AUG initiation codon, this polypeptide will not be produced as abundantly (the bacterial XGPRT experience¹³ fits this hypothesis). With respect to the data presented here (for example, with

Δ255J5), it seems that initiation at a particular AUG codon does not preclude initiation at a downstream AUG codon. Hence, if ribosomes do scan, initiation is curiously leaky. This problem is most notable in the Δ255L3 mRNA which possesses many potential initiation sites, as defined by the modified scanning model, upstream of the functional insulin initiation codon. In support of the modified scanning model, we find that some AUG triplets never function as initiation codons and that initiation at an AUG codon appears more efficient when this is the first AUG at the 5' end. We favour a model where eukaryotic ribosomes directly recognize certain AUG triplets which have favourable structures at the 5' end of a mRNA; proximity to the 5'-cap structure may modulate this recognition affecting the rate of polypeptide chain initiation.

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Periodic charge distributions in the myosin rod amino acid sequence match cross-bridge spacings in muscle

Andrew D. McLachlan & Jonathan Karn

Laboratory of Molecular Biology, University Postgraduate Medical School, Hills Road, Cambridge CB2 2QH, UK

The amino acid sequence of the rod portion of nematode myosin, deduced from the sequence of the unc-54 heavy chain gene of Caenorhabditis elegans, is highly repetitive and has the characteristics of an α -helical coiled coil. The molecular surface contains alternate clusters of positive and negative charge. Interactions between charge clusters on adjacent molecules could account for the observed spacings of the myosin cross-bridges in muscle. Calculations also suggest that the N-terminal third of the rod is only loosely associated with the thick filament backbone. Bending of the rod near the end of this region could allow the N-terminal section to act as a hinged arm during muscle contraction.

STRIATED muscle contains interdigitated lattices of thick and thin filaments^{1,2}. As first suggested by both H. E. and A. F. Huxley^{3,4}, it is thought that contraction results when the actin-containing thin filaments slide past the myosin-containing thick filaments. The myosin molecule is adapted to perform both a structural task, in the assembly of the thick filament, and enzyme reactions, which generate tension. The native molecule contains two heavy chains^{5,6} each of molecular weight (MW) $\sim 200,000$. Long regions of these chains nearest the carboxyl end form an α -helical coiled coil, known as the rod, and these rods form the backbones of the thick filaments. The myosin molecule also has two globular heads^{6,7} composed of the amino ends of each heavy chain combined with the four light chains⁵. The heads act as movable cross-bridges^{1,2} between the thick and thin filaments.

Interactions between the rod segments of the myosin molecules are necessary for the assembly of thick filaments. Although the structural details vary considerably in different organisms, the general features are conserved and these are outlined in Fig. 1a. The thick filament is bipolar and contains myosin molecules assembled in an antiparallel arrangement in the central 'bare zone', which is about 1,600 Å wide^{8,9}. Throughout the rest of the filament the rods form two parallel arrays in which the heads project away from the surface, with an axial stagger of ~ 143 Å between heads^{10–13} and a helix repeat in vertebrates of 429 Å. These spacings give rise to the familiar X-ray diffraction pattern of muscle^{10–12}. Proteolysis of thick filaments^{5,14} and isolated myosin suggests that the backbone of the thick filament is largely formed by interactions between the C-terminal two-thirds of the myosin rod (light meromyosin or LMM), while the N-terminal third of the sequence, or subfragment-2 (S-2) region, is only loosely attached. Bending of the myosin rod in the region of the S-2–LMM junction has been postulated^{11,15} as a critical feature for the movement of the myosin cross-bridge during contraction.

We describe here how features of myosin rod sequences may account for the patterns of assembly of myosin molecules into thick filaments. We have based our analysis primarily on the complete sequence of the rod of a myosin heavy chain¹⁶ of the soil nematode, *Caenorhabditis elegans*, which we have recently deduced from DNA sequence studies of the cloned *unc-54* gene^{17–19}, but comparison of the nematode sequences with myosin rod sequences from mammals and *Drosophila* suggests that the features of the rod which we take to be structurally important are indeed maintained in all striated muscle types.

Myosin rod sequences are conserved

The complete 1,117-residue amino acid sequence of the nematode myosin rod is shown in Fig. 2. The proteolytic cleavage site at the N-terminus of the rod (the S-1–S-2 junction) is well defined and lies close to the proline residue which seems to mark the N-terminal boundary of the regular helical rod sequence (see below). Aligned with the nematode sequence are partial amino acid sequences for rabbit S-2 and LMM determined by Elzinga and his colleagues^{20,21} using protein chemistry, as well as LMM sequences of rat and *Drosophila* deduced from cDNA sequence analysis of clones by Mahdavi *et al.*²² and S. Bernstein *et al.* (personal communication). The rod sequences are homologous along their entire lengths and show high levels of matching amino acids. For example, there are 42.5% matching amino acids between the nematode and rabbit sequences from the beginning of the rod to residue 463, and 48.1% matching amino acids between nematode and rat from residues 673 to the end of the rat rod sequence at residue 1,100. The two mammalian sequences are even more highly conserved and show 75.9% amino acid homology from residues 787 to 1,098. Most of the sequence alterations are conservative, particularly at positions in the molecule where charged or hydrophobic residues are preferred, so that the chemical characteristics of the sequences are preserved.

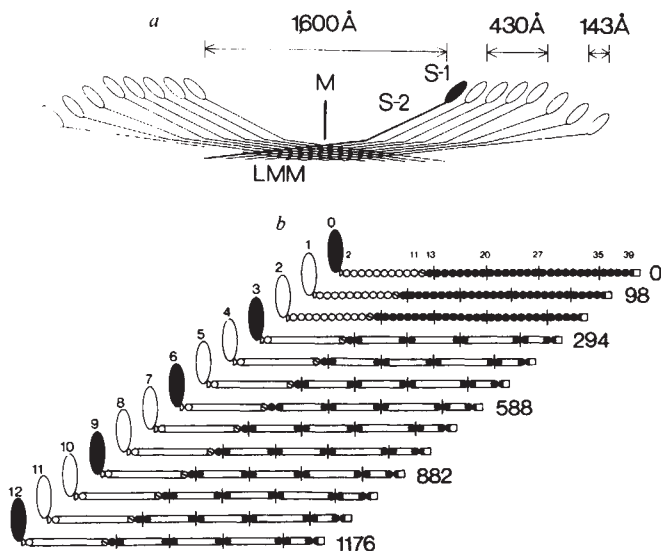


Fig. 1 *a*, Schematic drawing of the bipolar arrangement of myosin molecules in the thick filament. The filaments extend in both directions from the mid-line (M) and the globular heads form a regular array of cross-bridges with an axial separation of ~143 Å in vertebrate striated muscle and a helical repeat of 430 Å. The central bare zone, free of cross-bridges, is ~1,600 Å wide. Each molecule contains two chains (one only shown here) composed of the head, S-1, a helical rod-like arm, S-2, and a rod-like core segment, LMM. The width is not to scale. A typical complete vertebrate thick filament is ~15,700 Å long, with 49 levels of cross-bridges on each end and three cross-bridges emerging at each level^{2,8}. *b*, Parallel array of staggered myosin rods, drawn to show the relationship between the 143 Å (or 98-residue) and 430 Å (294 or 295 residues) displacements and the 28-residue zones in the amino acid sequence, as shown in Fig. 2. In rods numbered 0-2, open circles represent the zones in short S-2, ending within zone 12, and closed circles are zones in long S-2 or LMM. Vertical bars mark skip residues and the open square beyond zone 40 is the non-helical tailpiece. Rods 3-12 emphasize the half-staggered arrangement of skip residues. The diagram is a two-dimensional view of the stagger and should not be taken literally. The numbers at the right of the ends of the rods show the staggers in amino acid residues 0, 98, 294... A cross section through one such staggered array contains approximately 12 rods if S-2 is bound to the backbone or 8 rods when S-2 is bent away in zone 12. Vertebrate striated muscle probably has the equivalent of three such arrays in each cross-section.

28-Residue repeat units and helical structure of the myosin rod

The myosin rod sequence is highly repetitive and shows features typical of an α -helical coiled-coil protein. Between residues 2 and 1,088 the predicted α -helix probability profile²³ is high almost everywhere, with levels of 70-90% except for 15 local weak spots with a level below 50%. This whole region contains no proline. Beyond residue 1,088 the helix seems to end, leading into a tailpiece, Pro 1,095-Phe 1,117, with an apparently irregular secondary structure.

Many α -helical coiled-coil proteins²⁴⁻²⁶, especially tropomyosin^{27,28}, fibrinogen^{29,30} and α -keratin³¹, have a characteristic regular 7-residue pattern *a, b, c, d, e, f, g*, in which hydrophobic residues are concentrated at alternate intervals of three and four residues along the lengths of the chain at positions *a* and *d* (refs 24, 32). The residues at positions *a* and *d* form a closely packed hydrophobic interface or 'core' of knobs and holes between the two strands of the coiled coil³³. Fragments of the rabbit skeletal myosin rod sequence have already been found^{21,34} which show a marked 7-residue hydrophobic repeat, though less regular than that in tropomyosin²⁸. The histograms in Fig. 3 show how the complete nematode rod sequence has hydrophobic residues strongly concentrated in the core positions *a* and *d*. The helix surface is highly charged, with acidic and basic residues clustered mainly in the outer positions *b, c* and *f*.

Several fibrous proteins^{35,36} have longer repeated structural units which are important in the assembly and function of these molecules. The myosin rod shows strong evidence for a 28-

residue unit with a repetitive sequence as suggested originally by Parry³⁷ in his analyses of the rabbit myosin sequence determined by Elzinga *et al.*^{20,21,34}. Comparison of the sequence with itself shows that the entire helical sequence of the coil can be mapped out as a cyclical pattern of 28 amino acids. The statistical analysis of the pattern and its significance will be given elsewhere. We stress here two features which appear relevant to the rod structure. First, the repeat pattern is interrupted at four positions by the insertion of one extra residue which we call a 'skip residue'. As will be discussed below, these residues probably modulate the pitch of the coiled coil, a possibility previously proposed by Elzinga and Trus³⁸. Second, the distribution of the charges on the helix surface suggests that the rods are held in the thick filament structure by electrostatic forces between rods which lie side by side.

The positions of the skip residues are narrowly defined to within one or two turns of α -helix, and we have assigned them to residues 351, 548, 745 and 970, which are separated by regular intervening regions with lengths which are exact multiples of 28—196, 196 and 224, respectively. When aligned in this way, the coil region divides into 41 zones, 38 of which show a complete 28-residue cycle composed of four 7-residue sections with the characteristic coiled-coil pattern, beginning at position *d*. The 28 positions in a zone can thus be labelled 1*d*, 1*e*, ... 1*c*, 2*d*, 2*e*, ... 4*b*, 4*c*. With this convention the skip residues all occur at the end of a zone after position *c*, and the next zone begins at position *d*. The arrangement of repeat units and skip residues suggests that the whole sequence has evolved from a primitive 28-residue segment by a series of gene multiplication steps, perhaps involving a longer 197-residue structural unit in the later stages.

Each zone has a characteristic pattern of hydrophobic residues and a remarkable distribution of charges. The histograms in Fig. 3 show these patterns, which are drawn more realistically on a helical net diagram in Fig. 4. A typical 28-residue zone divides into six alternating bands of positive and negative charge which are spaced so that the strongest peak of positive charge (position 1*b*) is exactly 14 residues (or one half-zone) away from the strongest peak of negative charge (position 3*b*). A Fourier analysis of the charge distribution shows very intense peaks at periods of 28 and 28/3 residues in both the acidic and basic amino acids. Parry detected some of these peaks in earlier sequence fragments³⁷.

The hydrophobic residues are not uniformly distributed throughout the 28-residue zones and some positions such as 1*a* and 4*d* are particularly weak, because of the displacement of hydrophobic residues by charged amino acids. Lysine and arginine often appear in the *a* core positions (31 examples). The negatively charged side chains of aspartic and glutamic acid are, however, totally excluded from position *a* and are rare in position *d* (15 examples) except near the maxima of the negative charge pattern.

Although individual zones differ, the character of a typical 28-amino acid zone may be summed up in a 'consensus' sequence derived by taking the most common residue at each position:

<i>d</i>	<i>e</i>	<i>f</i>	<i>g</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>	<i>g</i>	<i>a</i>	<i>b</i>	<i>c</i>	
L-E-K-Q-K-K-K-L-E-Q-E-L-E-E	(1-14)													
L-Q-E-Q-L-D-E-E-E-R-A-L-A-K	(15-28)													

Skip residues and the coiled-coil pitch

The skip residues appear at homologous positions in each of the species studied so far, and break the 7-residue pattern of the coiled-coil sequence. We built space-filling models of the skip regions and several comparable 56-residue lengths of the regular coil to test whether the extra residue produced a complete break in the coiled coil or merely a minor deformation. Reasonable limits for the pitch of the regular supercoil range

Figure 5 shows how the net electrostatic interaction between two parallel rod sequences depends on the axial stagger. A pair of molecules was treated as two linear arrays of charged and uncharged amino acids shifted successively by 0, 1, 2, ... n residues. Two charges were considered to interact if they lay nearly opposite one another, within a range of 0, ± 1 , ± 2 residues. In these calculations only the acidic and basic amino acids were considered and the coil was taken to have a length of 1,094 residues, ignoring the non-helical tailpiece, at residues 1,095–1,117. The energy oscillates strongly with stagger, showing a 28-residue period, and has peaks close to the odd multiples of 14. The amplitude decreases as the molecular overlap is reduced, but not uniformly, and several peaks stand out. The most prominent are at odd multiples of staggers of 98—for example, 98, 294 (98×3) and 490 (98×5). A stagger of 98 residues or $3\frac{1}{2}$ complete zones would correspond to a distance of 145.5 Å, assuming a helical rise of 1.485 Å per residue⁴³ in the α -helical coiled coil. This distance is close to the cross-bridge translation of 143–146 Å observed in muscle. A small correction is required for an average of one skip residue every 196 residues, so that the theoretical step length would be 98.5 residues or 146.3 Å. A further unknown correction is needed if the rods bend, or if they run at a small angle to the thick filament axis. The interactions seen at staggers that are odd multiples of 98 allow for several kinds of nearest neighbour in the close-packing of myosin rods in three dimensions. Thus, the stagger of 294 residues accounts for the interactions between ideal straight molecules translated by 436.5 Å or between real molecules at the axial helical repeat distance of 430 Å seen in muscle. Similarly, the interactions at a 490-residue stagger reflect potential interactions between molecules further apart in the helix.

This overlap calculation does not show which portions of the rod interact most strongly, nor whether attractions and repulsions are equally important. A more detailed picture was built up by calculating local interactions between shorter segments of staggered rods with a certain span length. Trials showed that the clearest results were obtained when attractions and repulsions had equal weights and when interactions were averaged over long spans of 99–589 residues.

Significant interactions appear at most staggers which are half-integer multiples of 28, especially in the first half of the molecule, but the strongest and most persistent interaction is at a stagger of 98. In Fig. 6 the interaction density summed over a span of 99 residues is plotted along the length of the rod for staggers of 294, 100, 98 and 96 residues. The favourable interactions for a stagger of 98 stand out clearly. The strong peak centred on residue 320 corresponds to the attraction between residues 271–369 of one rod and 173–271 of its neighbour, displaced 98 residues towards the carboxyl end. The pairing persists strongly down to the shoulder near residue 880. The interactions at a stagger of 294 residues are generally weaker and they appear mainly in the second half of the molecule. The high plateau from residues 740–1,008 in Fig. 6 corresponds to a strong averaged interaction in the LMM fragment between residues 691–1,057 and 593–959, with a stagger of 294 residues which corresponds to the helix repeat distance in vertebrate muscle.

Search for a hinge between S-2 and LMM

It has been suggested that myosin contains flexible sections or hinges. A hinge in the rod between S-2 and LMM could allow the cross-bridge to move in or out from the thick filament backbone^{10,15}. Electron microscope observations of sharp bends in isolated myosin rods^{7,44} support this notion. Also, proteolysis of myosins from a wide variety of species^{5,6,14} shows that the rod tends to cleave at narrowly localized points, which may reflect the presence of hinges. In rabbit skeletal muscle either a 37,000-MW fragment (short S-2) or a 59,000-MW one (long S-2) can be cleaved from the amino end of the rod. Analysis of the rabbit fragments^{21,38,45,46} suggests that the C-terminus of short S-2 is homologous to a region in the nematode between

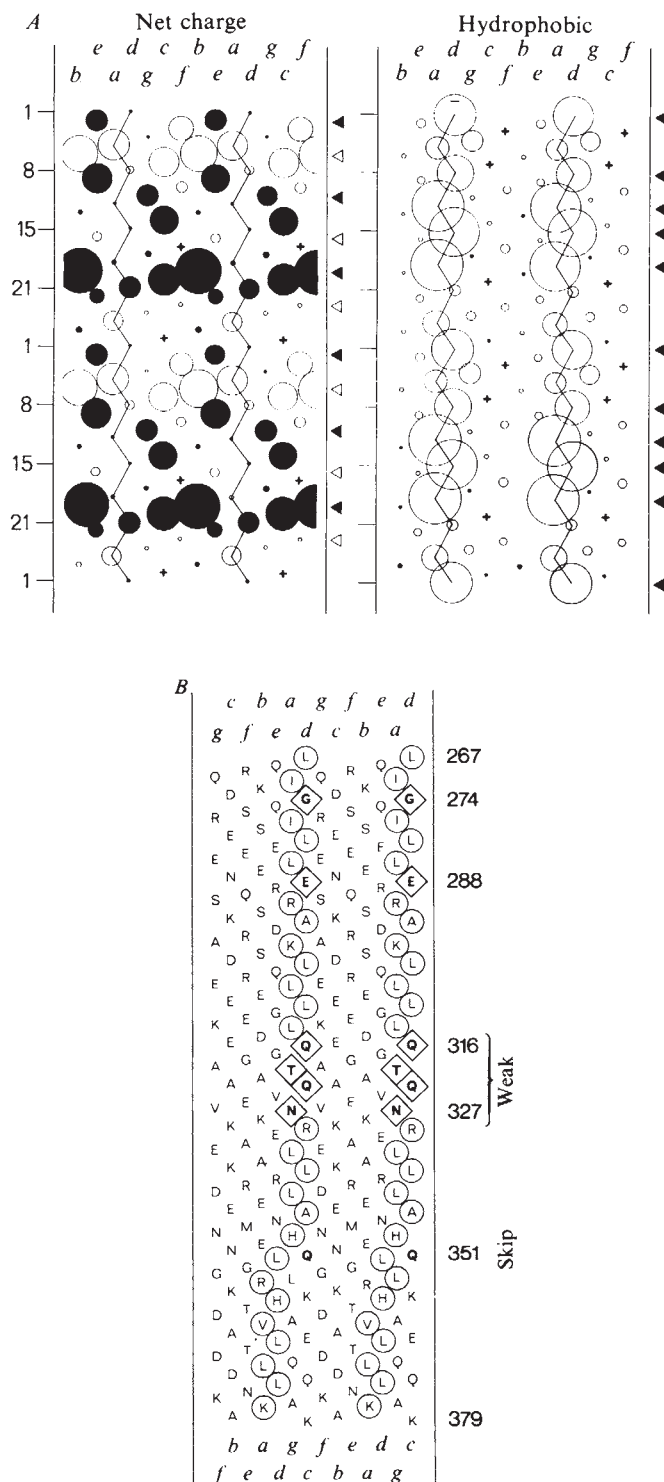


Fig. 4 A, charge distribution and hydrophobic residues for the averaged 28-residue zone plotted on the supercoil surface of two paired α -helices. A section of coil 56 residues long has been projected onto a cylinder and unrolled so that each section of sequence appears twice side-by-side, first on one helix and then on the other. The sequence reads across from right to left with a slight downward slant and is drawn with a pitch of $3\frac{1}{2}$ residues per turn. Inner positions a, d are connected by a zig-zag line. Circles have radii proportional to the numbers in Fig. 3, with white and black to represent positive and negative charges. A (+) sign marks points of zero net charge. Triangles at the side mark six bands of charge in the typical zone. Hydrophobic residues are plotted in a similar way with triangles to mark the major sites and (+) signs to mark forbidden positions. B, helical net of part of the coiled-coil sequence to illustrate the interrupted pattern of hydrophobic residues in the core. Hydrophobic or basic amino acid side chains are ringed, while diamonds enclose acidic, polar or glycine side chains, which are likely to weaken the core. At skip residue Gln 351 the pattern shifts across the helix surface. Residues 316–327 may form an unusually weak part of the coil and they lie near the short S-2–LMM junction.

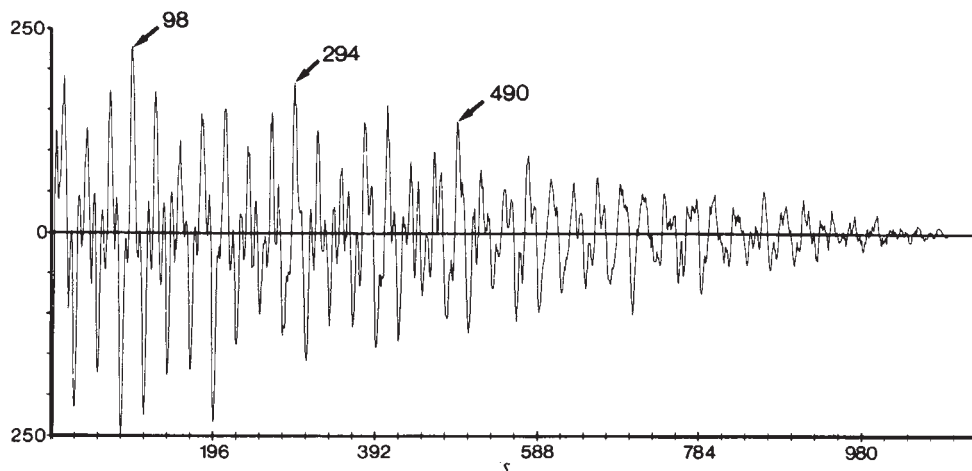


Fig. 5 Charge interactions between parallel rods 1,094 residues long. The stagger, s , is plotted horizontally and the net number of interactions (attractions minus repulsions) vertically. Amino acids are counted within an axial range of ± 2 residues, taking each rod as a single chain, because only one side at a time can interact with its neighbour. Note the peaks at staggers which are odd multiples of 98.

lysines 312 and 328, which would give a molecular weight of between 36,173 and 37,783. The remainder of the rod, possibly the longest LMM fragment, would then have a MW of 91,386 or 89,776. Lu and Wong⁴⁵ have also demonstrated that fragments of rabbit myosin homologous to residues 326–386 are present in long but not short S-2. The long S-2–LMM junction⁴⁶ is near Lys 517. Cleavage at this point would lead to a 59,313-MW fragment and a corresponding form of LMM with MW 68,246.

There is no part of the nematode rod or the vertebrate sequence fragments which seems at all likely to form a specific non-helical molecular hinge distinct from the coiled coil. The short S-2 region and LMM are predicted to be α -helical to the same extent and the 28-residue repeat pattern is strong in both regions. The total net charge differs little. The skip regions do not seem to correspond to the hinges defined by proteolysis. The first skip residue, at position 351, and the second at 548 lie well to the C-terminal sides of the likely cleavage sites for short and long S-2. There is no evidence for specific cleavages near the third and fourth skip residues.

We searched the nematode sequence for potential weak spots where several consecutive missing hydrophobic residues in the core might disturb the coiled-coil structure. The sequences in Fig. 2 and the histograms in Fig. 3 suggest that Ala can substitute satisfactorily for Leu, Val or Ile in the core positions a and d , while the hydrophobic basic groups Arg and Lys are tolerated more readily than Asp and Glu. Therefore, we looked for regions with polar, negatively charged, or glycine residues in positions a and d . With this rule the weakest sections of the coil seem to be positions 316–327, with four consecutive defects, and 176–183 and 823–830 with three each. The section 316–327, which is one of the most pronounced of these potential weak points, lies close to the point of division between the short and long S-2 fragments in rabbit^{45,46}. We have also obtained (J. K. and L. Barnett, unpublished) the sequences of two other nematode heavy chains in the region 316–327. In one gene the sequence is identical to *unc-54*, but the second shows an apparently stronger structure in which Leu replaces Thr 320 and strengthens the core.

The freedom of movement of the S-2 region must also depend on its interactions with the rest of the filament. Our analysis of the electrostatic interactions between parallel rods (Fig. 6) suggests that the N-terminal part of the short S-2 region has a weakened attraction, or even repulsion, for other molecules aligned with staggers close to 98 residues, whereas the middle section, residues 173–271, has a strong attraction for residues 271–369 of an adjoining molecule. At certain staggers the tip of S-2 can be attracted to other sections of the myosin rod. A particularly strong interaction appears when the stagger is 95 or 96.

Our analysis suggests that the S-2 region is held against the rest of the thick filament by a comparatively weak 'electrostatic spring' which allows the tip of the rod to bend outwards under stress. Residues 316–327 may be a 'soft spot' in the coiled coil

where the α -helix can bend or even unravel and extend locally⁴⁷ to accommodate the movement.

Structure of thick filaments

The high density of charged groups on the outer surface of the myosin rod and the lack of hydrophobic groups on the surface suggest that ionic interactions are by far the most important forces involved in the assembly of thick filaments. We have seen that the rod sequence is made up of structurally similar repeat units which are 28 residues (or 41.6 Å) long, which have a characteristic charge distribution and attract one another strongly when they are half-staggered by half-integer multiples of their length. Our one-dimensional model calculations suggest that direct interactions between complementary charges on parallel rods staggered by 98 or 294 residues may account for the 143 and 430 Å axial spacings found in muscle. Our result

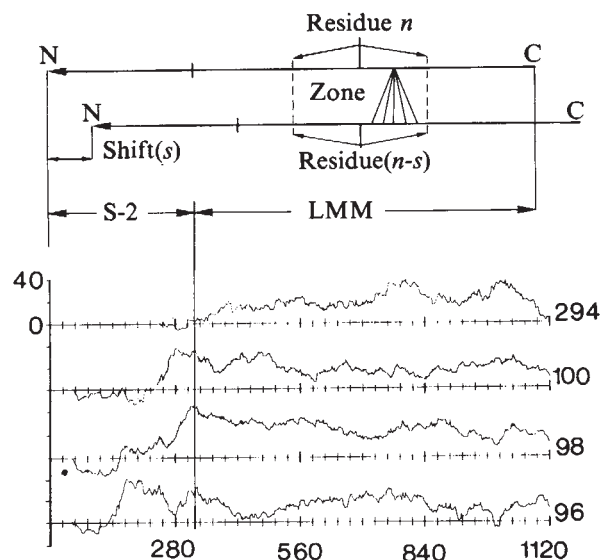


Fig. 6 The distribution of charge interactions along the length of a pair of parallel rods. The upper rod is fixed and the lower rod has been shifted s residues to the right. The curves are drawn for shifts of 96, 98, 100 and 294 residues and the positions of the rods shown here correspond to the 98-residue shift. The height of the graph under residue n of the upper rod is equal to the sum (attractions – repulsions) of a 99-residue section centred on residue n and the adjacent 99-residue section of the lower rod centred on residue $(n-s)$. When residue n nears the end of the sequence the 99-residue active zone becomes shortened and the interactions gradually decrease at each end. Thus, the top curve for a shift of 294 residues reaches its peaks between residues 343 and 1,045. The interactions are scored in the same way as in Fig. 5. Inside each 99-residue section the charge pairs are counted within an axial range of ± 2 residues. The relationship between the zone and the scoring is shown schematically in the upper diagram (not to scale). The S2–LMM junction has been placed at residue 322 and refers to the upper rod.

that the 294-residue stagger leads to strong attractions in the LMM segment agrees with some observations on LMM paracrystals^{48,49}. The 430 Å stagger often coexists in a phase equilibrium with the 143 Å stagger. The 143 and 430 Å spacings associated with the myosin molecule are both close to half-integral multiples of 286 Å and suggest³⁷ that the long spacings in muscle are produced by electrostatic interactions between larger-scale structural units ~196 or 197 residues long, which form a variety of half-staggered fibrous arrays. The two 197-residue sections of myosin rod, 351–547 and 548–744, bounded by the first three skip residues, may therefore be discrete structural units in the assembly of the thick filament. If this type of structural scheme is correct, it would suggest that paramyosin⁵⁰, which assembles with a long spacing of 725 Å (2½ times 290 Å), also contains 28-residue zones and skip residues, and that the shorter 290 Å network spacing in paramyosin results from a combination of opposed half-integer spacings of 725 and 435 Å.

We have not yet extended our calculations to analyse the three-dimensional packing of myosin rods in the thick filaments. All such models² depend crucially on the pitch of the supercoil, and in myosin the presence of skip residues which probably modulate the pitch is an added complication. Two funda-

mentally different types of attraction are possible; direct salt bridges⁵¹ or indirect ion-bridged couplings between clusters of like charges⁵². Packing schemes which only allow direct salt bridges are quite restricted. A cross-section through the filament at each level must contain a lattice of alternating positive and negative charges with nearest neighbours formed into even-membered sub-assemblies of 2, 4, 6, . . . two-stranded myosin rods. The simplest regular assemblies of this type have square or hexagonal cross-section lattices. The presence of 28-residue zones throughout the sequence suggests that the packing may originally have evolved from a regular crystalline lattice in which amino acids separated by 28 residues occupied equivalent sites.

Finally, we note that numerous mutants of the *unc-54* gene have been isolated which show defects in the thick filament assembly. Analysis of the sequence alterations in these strains should provide an experimental framework for testing the ideas presented here.

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The effects of α chain mutations *cis* and *trans* to the $\beta 6$ mutation on the polymerization of sickle cell haemoglobin

Ruth E. Benesch, Suzanna Kwong & Reinhold Benesch

Department of Biochemistry, Columbia University, College of Physicians and Surgeons, New York, New York 10032, USA

*The solubility of polymers formed by haemoglobins with one β^S chain and one mutant α chain, either *cis* or *trans* to each other, has revealed three distinct regions on the surface of the tetramer which participate in intermolecular bonding within the haemoglobin S fibre.*

IT has become increasingly clear that the polymer formed from concentrated solutions of deoxygenated sickle cell haemoglobin (HbS) is a multi-stranded helix, the basic unit of which is the double-stranded structure described for HbS crystals by Wishner *et al.*¹. This is supported by the spontaneous transfor-

mation of fibres into crystals that show the same diffraction pattern as those studied by Wishner *et al.*, which has been demonstrated for fibres stored for very long periods of time², and also for fibres stirred to accelerate their transformation to crystals^{3–5}—in which case a considerable polymorphism of the

intermediate structures formed during crystallization can be observed.

The link between the HbS fibre structure and that of the Wishner double strand is also supported by the demonstration that many of the mutations that interfere with HbS gelation are located at residues that form contacts between the haemoglobin molecules in the Wishner crystal⁶⁻⁹. The significance of the double strands as the basic unit from which the fibres are constructed is also illustrated by the observation that specific pairs of strands are missing from the fibre structure in certain conditions. This was found in minor forms of HbS fibres¹⁰ as well as in the principal form of the fibres of the double mutant haemoglobin $\alpha_2^{\text{Sealy}}\beta_2^{\text{S}}$ (ref. 11).

In HbS crystals the molecules are arranged in paired strands with tetramers in adjacent strands staggered by half a molecular diameter¹. The lateral contact between members of a pair involves the HbS mutation in only one of the β chains of a tetramer—a hydrophobic bond between valine 6 of the β_2 chain of a tetramer in one strand with residues on the haem pocket of the β_1 chain of a molecule in the adjacent strand. It follows that in the crystal (and therefore also in the fibre) the two β chains, and hence also the two α chains, are not equivalent and that residues on the surface of the α_1 and the α_2 chain will be involved in different groups of intermolecular contacts. The different role of the two β chains in the polymerization was first demonstrated in solution by the effect of HbA on the polymerization of HbC-Harlem¹², and later by the finding that tetramers containing only one β^{S} chain can form gels^{13,14}.

As the structure of the fibres of deoxy HbS has not been solved unequivocally, several models have been proposed for

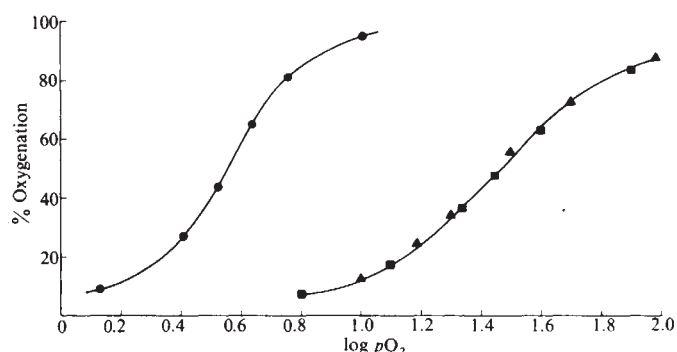


Fig. 1 The effect of the cross-link on oxygenation curves. These were determined spectrophotometrically as described elsewhere^{29,30}. ●, HbS; ▲, cross-linked HbS; ■, cross-linked HbAS. 0.05 M bis Tris buffer, pH 7.3, 0.1 M chloride, 20 °C.

fibres composed of four¹⁵, seven^{10,16,17} or eight¹⁸ 'Wishner double strands'. Of these, the eight-stranded one is the most unlikely, having been proposed before the non-hollow structure of the fibres was recognized. The 16-stranded model was proposed on the basis of the fibre to crystal transformation^{3,18}, but only the 14-stranded structure results from direct image reconstruction of the fibre itself^{10,16,17}.

In such a multi-stranded helix at least three classes of intermolecular contacts can be distinguished: (1) The axial contact between molecules within a single strand. These contacts will be similar in all strands throughout the fibre. (2) The lateral contact between members of a double strand, described above. Again, this contact will exist throughout the structure, and $\beta_1,6$ valines do not participate. (3) A much more diverse group of contacts are those that hold the double strands together to form the multi-stranded helix. They are not only much less well characterized, but are necessarily of several different kinds, particularly in the case of the 14-stranded structure. These contacts must be different from the inter-pair ones in the crystal, and they are, of course, especially relevant for the stability of the fibre structure.

Double mutants, containing the β^{S} mutation and an additional one on the α chains, have previously been used to define regions on the α chain that participate in the intermolecular contacts⁶⁻⁸. As pointed out by Nagel *et al.*⁹, the conclusions that can be reached by this approach are limited from a stereochemical point of view, as both α chains and both β chains are substituted in these tetramers.

As only one of the β_6 valines participates in the bonding between the two partners of a double strand, the effect of an α chain mutation will depend on its position relative to the contact-forming β^{S} chain (β_2). With this in mind we have prepared tetramers containing only one β^{S} chain (the other one being β^{A}) so that an α chain mutation could be placed either on the same dimer as the β^{S} mutation (*cis*) or on the opposite one (*trans*). Because such haemoglobin tetramers, consisting of two different $\alpha\beta$ dimers, disproportionate to homogeneous ones during aerobic chromatography, they were isolated by using a specific covalent cross-link between the β chains^{19,20}.

The results presented here show that these molecules could be used to test some of the predictions which emerge from the proposed models of the sickle fibre.

Preparation of mixed tetramers

The general approach was to mix equal amounts of the two haemoglobins in aerobic conditions to allow for $\alpha\beta$ dimer exchange which results in a mixture containing 50% of the hybrid and 25% of each of the parent haemoglobins. The mixture was deoxygenated to freeze the equilibrium, and an intra-dimeric cross-link was introduced by reaction with 1.4 mols of 2-nor-2-formylpyridoxal 5' phosphate per mol of haemoglobin, followed by reduction with NaBH_4 as described previously^{19,20}.

Table 1 Effect of $\alpha 16$ and $\alpha 47$ mutations on gel solubility

Expt	c_{sat} (g per 100 ml)	γ	a_{sat} (g per 100 ml)	$\frac{(a_{\text{sat}})\alpha\text{mutant}}{(a_{\text{sat}})\alpha\text{A}}$
<i>a</i>				
$\beta^{\text{S}}\alpha^{\text{A}}$	14.6 ± 1.3 (22)	3	44	—
$\beta^{\text{S}}\alpha^{\text{A}}$				
$\beta^{\text{S}}\alpha^{\text{I}}$	26.6 ± 1.2 (10)	15	405	9.2
$\beta^{\text{S}}\alpha^{\text{I}}$				
$\beta^{\text{S}}\alpha^{\text{Sealy}}$	20.2 ± 1.3 (10)	6.5	135	3.1
$\beta^{\text{S}}\alpha^{\text{Sealy}}$				
<i>b</i>				
$\beta^{\text{S}}\alpha^{\text{A}}$	12.3 ± 0.5 (6)	2.5	33	—
$\beta^{\text{S}}\alpha^{\text{A}}$				
$\beta^{\text{S}}\alpha^{\text{I}}$	22.8 ± 0.1 (2)	8.5	194	5.9
$\beta^{\text{S}}\alpha^{\text{I}}$				
$\beta^{\text{S}}\alpha^{\text{Sealy}}$	14.9 (1)	3.1	46	1.4
$\beta^{\text{S}}\alpha^{\text{Sealy}}$				
<i>c</i>				
$\beta^{\text{S}}\alpha^{\text{A}}$	20.0 ± 1.8 (11)	5.5	110	—
$\beta^{\text{A}}\alpha^{\text{A}}$				
$\beta^{\text{S}}\alpha^{\text{I}}$	31.1 ± 0.7 (2)	29.5	917	8.3
$\beta^{\text{A}}\alpha^{\text{I}}$				
$\beta^{\text{S}}\alpha^{\text{Sealy}}$	21.0 ± 0.3 (3)	6.6	139	1.3
$\beta^{\text{A}}\alpha^{\text{Sealy}}$				

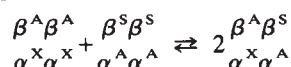
HbA was obtained from normal human blood as described previously²³. HbS ($\alpha_2\beta_2^{\text{Glu} \rightarrow \text{Val}}$), HbSealy ($\alpha_2^{47\text{Asp} \rightarrow \text{His}}\beta_2^{\text{S}}$) and Hb^I ($\alpha_2^{16\text{Lys} \rightarrow \text{Glu}}\beta_2^{\text{S}}$) were isolated from the blood of heterozygous donors using the chromatographic system of Abraham *et al.*²⁴ on a preparative scale²⁵. Polymerization was induced by reduction with two equivalents of deoxygenated dithionite per haem, the gels were centrifuged and the haemoglobin concentration was determined in the supernatant as described elsewhere²⁶. For many of the experiments the volume of haemoglobin solution necessary was decreased to 80–100 μl by using centrifuge tubes 45 mm in length made from Pyrex capillary tubing (i.d. 2 mm, o.d. 5 mm). The supernatant concentration (c_{sat}) is independent of the initial concentration²⁵. Because these concentrated solutions are highly non-ideal, c_{sat} must be multiplied by the activity coefficient (γ)²⁷ to obtain a thermodynamic measure of the polymerizing tendency (a_{sat}).

Table 2 Gel solubility of tetramers with *cis* and *trans* $\alpha 16$ mutations

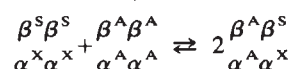
	c_{sat} (g per 100 ml)	γ	a_{sat} (g per 100 ml)	$(a_{\text{sat}})_{\alpha \text{ mutant}}$ $(a_{\text{sat}})_{\alpha A}$
$\left[\begin{smallmatrix} \beta^S \alpha^A \\ \beta^A \alpha^A \end{smallmatrix} \right]$	20.0 $\pm$ 1.8 (11)	5.5	110	—
$\left[\begin{smallmatrix} \beta^S \alpha^I \\ \beta^A \alpha^I \end{smallmatrix} \right]$	31.1 $\pm$ 0.7 (2)	29.5	917	8.3
<i>cis</i> $\left[\begin{smallmatrix} \beta^S \alpha^I \\ \beta^A \alpha^A \end{smallmatrix} \right]$	31.3 $\pm$ 0.1 (3)	31	973	8.8
<i>trans</i> $\left[\begin{smallmatrix} \beta^S \alpha^A \\ \beta^A \alpha^I \end{smallmatrix} \right]$	19.7 $\pm$ 0.2 (3)	5.5	108	1.0

Following the NaBH_4 reduction, all haemoglobins were converted to the metcyanide form by oxidation at pH 7.0 and room temperature with 1.2 equivalents of $\text{K}_3\text{Fe}(\text{CN})_6$ per haem in the presence of 2.0 equivalents of KCN. Ferri cyanide and ferrocyanide were removed by passage through Sephadex G-25 in 0.1 M phosphate buffer pH 7.3 containing 10^{-2} M KCN. The three cross-linked species (the hybrid and the two parent haemoglobins) were then separated from uncross-linked haemoglobins by passage of the mixture through a 4×52 cm Sephadex G-100 column in 1 M MgCl_2 , 0.1% Tris pH 7.0, 10^{-2} M KCN at 18 ml h^{-1} (ref. 28). The isolation of the desired cross-linked haemoglobins was again accomplished by the Abraham method²⁴ using sectional columns²⁵. This method resulted in excellent separations of all the components of the mixtures except in the case of the *trans* isomer of the Hb^{Sealy} :HbS hybrid. Since HbS, Hb^{Sealy} and the hybrid all have the same charge in neutral solution, a lower pH to protonate the $\alpha 47$ histidine of Hb^{Sealy} was used to resolve this mixture. This was done by chromatography on CM-Sephadex in 0.05 M phosphate buffer pH 6.0 with a gradient of NaCl from 0 to 0.1 M. After elution all haemoglobin solutions were dialyzed to equilibrium against 0.1 M phosphate buffer pH 7.3 containing 0.01 M KCN. They were then concentrated as described previously²⁵.

(1) *Trans* tetramers, with the α and β chain mutations on different $\alpha\beta$ dimers. These are made by mixing HbS and the α mutant haemoglobin, thus:



(2) *Cis* tetramers, with the α and β chain mutations on the same $\alpha\beta$ dimer. The starting material for this kind of tetramer was the double mutant, $\alpha_2^X \beta_2^S$, prepared by previously described methods⁶⁻⁸. The *cis* tetramer is formed by subunit exchange of this double mutant with HbA, thus:



The structure of these mixed tetramers is unequivocally determined by their method of preparation, as the dissociation of haemoglobin in neutral solution does not proceed beyond the $\alpha\beta$ dimer stage.

The pyridoxal phosphate dialdehyde used throughout this investigation for stabilizing the mixed tetramers provides a single covalent bridge between the N-terminal amino group of one β chain and lysine 82 of the other β chain, across the polyphosphate binding site²¹. This cross-link greatly lowers the

O_2 affinity (Fig. 1) to values similar to those found in the presence of excess inositol hexaphosphate. Furthermore, the cross-link does not appear to alter the structure of the fibres, as cross-linked HbS fibres are typical of those of normal HbS in the presence of organophosphates (Fig. 2).

Polymerization studies

The effect of the two mutations in the α chain ($\alpha 16$ and $\alpha 47$) on the gel solubility of tetramers that also carry the β^S mutation is compared in Table 1. Three types of tetramers were initially studied: (1) Uncross-linked tetramers in which both α chains and both β chains are substituted. (2) The same tetramers cross-linked between the β chains. (3) Cross-linked tetramers in which both α chains are mutant ones, but only one β chain is β^S .

It is clear that in all cases the solubilizing effect of the $\alpha 16$ substitution far exceeds that at $\alpha 47$. Both these mutations were previously reported to increase the solubility in concentrated phosphate, but with their relative effectiveness reversed. This was probably due to the influence of the high ionic strength used in this assay on the electrostatic interactions responsible for these contacts⁶. A comparison of the results for (2) and (3) shows that the tetramers containing only one β^S chain behave similarly to the ones with two β^S chains except for a higher solubility. The somewhat lowered gel solubility of cross-linked compared with uncross-linked HbS (Table 1) also shows that this modification does not interfere with polymerization but actually favours it, probably because it fits so well into the deoxyconformation of the central cavity²¹.

The results in Table 2 show the dramatic difference in the effect of the $\alpha 16$ mutation in the *cis* compared with the *trans* configuration. The tetramer in which both mutations are present on the same $\alpha\beta$ dimer (*cis*) is almost 10 times as soluble as the *trans* tetramer, which has the same solubility as the one in which both α chains are normal (Table 2). As substitution of glutamate for lysine at position 16 in the *trans* α chain has no effect on polymerization, the mutation in the *cis* position must be responsible for the whole solubilizing effect observed when both α chains are mutant ones.

The corresponding data for the $\alpha 47$ mutation are shown in Table 3. In this case substitution of mutant for normal α chains in both positions affects polymerization, but in opposite directions. While the mutation in the *cis* α chain causes an increase in solubility, the same substitution in the *trans* α chain actually decreases it (Table 3). The extent to which these two opposing effects neutralize each other evidently depends on the kind of tetramer into which the two α chain mutations are introduced. In uncross-linked HbS the solubilizing effect prevails (Table 1a) whereas in the cross-linked ones the two effects largely cancel each other (Table 1b, c).

Discussion

The striking difference in the solubility of the *cis* and *trans* isomers of both mutations testifies to the different role that each of the α chains has in the formation of the HbS fibre.

In the case of the $\alpha 16$ mutation, substitution of the normal

Table 3 Gel solubility of tetramers with *cis* and *trans* $\alpha 47$ mutations

	c_{sat} (g per 100 ml)	γ	a_{sat} (g per 100 ml)	$(a_{\text{sat}})_{\alpha \text{ mutant}}$ $(a_{\text{sat}})_{\alpha A}$
$\left[\begin{smallmatrix} \beta^S \alpha^A \\ \beta^A \alpha^A \end{smallmatrix} \right]$	20.0 $\pm$ 1.8 (11)	5.5	110	—
$\left[\begin{smallmatrix} \beta^S \alpha^{\text{Sealy}} \\ \beta^A \alpha^{\text{Sealy}} \end{smallmatrix} \right]$	21.0 $\pm$ 0.8 (3)	6.6	139	1.3
<i>cis</i> $\left[\begin{smallmatrix} \beta^S \alpha^{\text{Sealy}} \\ \beta^A \alpha^A \end{smallmatrix} \right]$	23.8 $\pm$ 0.5 (2)	9.8	233	2.1
<i>trans</i> $\left[\begin{smallmatrix} \beta^S \alpha^A \\ \beta^A \alpha^{\text{Sealy}} \end{smallmatrix} \right]$	12.7 $\pm$ 0.8 (5)	2.7	34	0.3

Experimental details were as for Table 2.

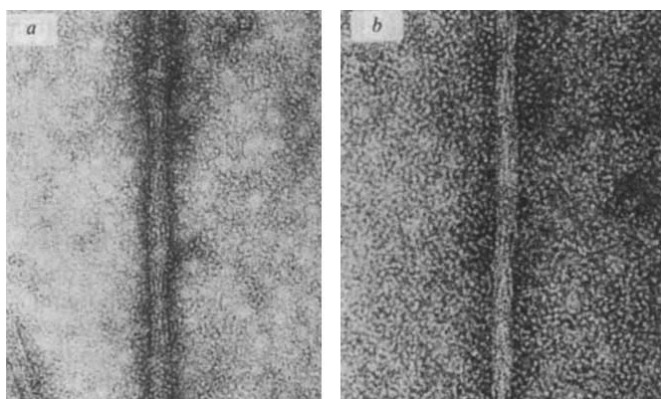


Fig. 2 Electron micrographs of HbS and cross-linked HbS fibres. a, HbS; b, cross-linked HbS. 2% Phosphotungstic acid. $\times 280,000$.

lysine by a glutamate in the α_2 chain causes a very large increase in gel solubility, whereas the same substitution in the α_1 chain has no effect at all. According to Wishner *et al.*¹ lysine α_{216} is situated in the axial contact between the molecules along a single strand, and the normal attraction between this lysine and glutamate β_{122} in the next molecule would then be replaced by a repulsion in the mutant. Lysine α_{116} , on the other hand, is not involved in any of the contacts of the Wishner-Love structure. Our results with the α_{16} mutation therefore strongly support the Wishner-Love double strand as a basic unit of the HbS fibre.

The significance of the α_{16} locus for the polymerization of HbS raises the possibility of using it as a receptor site for antisickling modifications. In this connection it is of interest that Acharya and Manning found that lysine α_{16} was the most reactive residue in the α chain towards glyceraldehyde²².

The situation is quite different for the α_{47} mutation. Neither α_{147} nor α_{247} is involved in intra double strand contacts¹, but in both cases substitution of the normal aspartate by a histidine

changes the stability of the fibre (Table 3). The observed effects must therefore be due to contacts between the double strands. Since these results thus identify two separate regions on the tetramer, (α_{147} and α_{247}) as being involved in inter double-strand contacts, they provide much needed constraints for the orientation of the double strands within the HbS fibre. It was shown earlier that the presence of this mutation in both α chains of a HbS tetramer leads to fibres with a loss of two specific pairs of strands, 'capped' by a ring of 16 new strands¹¹. Hopefully electron microscopy of the *cis* and *trans* isomers will resolve the question of the relative roles of the mutation in the two different α chains in causing these structural alterations.

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LETTERS TO NATURE

Discovery of a very red nucleus in the radio elliptical IC5063 (PKS2048-57)

D. J. Axon*, Jeremy Bailey† & J. H. Hough‡

* Department of Physics and Astronomy, University College London, Gower Street, London WC1E 6BT, UK

† Anglo-Australian Observatory, PO Box 296, Epping, New South Wales, Australia

‡ School of Natural Sciences, Hatfield Polytechnic, Hatfield, Hertfordshire AL10 9AB, UK

We report here the discovery of an extremely red IR source in the nucleus of the elliptical galaxy IC5063 (PKS2048-57). The observed (2.2-3.8) μ m colour index within a 5 arc s aperture is 2.1 mag while the colours at shorter wavelengths and in the 5-10 arc s annulus around the nucleus are close to those of normal elliptical galaxies. The IR excess could arise from a BL LAC type nucleus with a steep non-thermal spectrum ($\alpha \sim -4.5$ defined by $F \propto \nu^\alpha$), similar to the recently identified 'red QSOs' typified by 1413+135 (refs 1, 2).

Low luminosity BL Lac objects are predominantly associated with the nuclei of elliptical galaxies which possess flat radio spectra for example, Mkn 421 (refs 3, 4). Many of the nearby E/S0 galaxies have similar radio properties, suggesting that they may also contain nuclei of this type^{5,6}. There are, however, good observational reasons for suspecting that most of this population would not have been recognized as BL Lac objects. The presence of a nuclear source embedded in a bright elliptical galaxy could be almost completely hidden in the visible and near IR by the stellar component. This would arise either

because it was of low luminosity like Mkn 421 or if it had a steep non-thermal spectrum similar to 1413+135, even if the spectrum only steepened at much shorter wavelengths. As most interest has centred on the stellar populations of elliptical galaxies there has been little attention directed at wavelengths longer than 2.2 μ m where radiation from the nuclei is expected to dominate.

NGC1052 is probably the best known example of a radio elliptical which could fit into this category with its large 10- μ m excess⁷ and its recently observed near IR polarization⁸. Other galaxies whose excess 10- μ m fluxes⁹ may qualify them as objects of this type are M87, NGC3894 and NGC4552.

The existence of 'BL Lac' nuclei in the class of active gas-rich ellipticals would be particularly important. These ellipticals are characterized at one extreme by the galaxies NGC1052 and PKS0349-27, which show shock excited line spectra^{10,11} and IC5063, PKS2158-380 at the other, whose optical spectrum is more likely to be due to photoionization by a non-thermal nuclear source^{12,13}. A detailed knowledge of the shape of the non-thermal spectrum of the nuclei of these galaxies would be a vital step in understanding both the physical processes at work in them, and their relationship to the origin of radio galaxies.

Observations of IC5063 and the three other active ellipticals mentioned above (NGC1052, PKS2158-380 and PKS0349-27) were obtained on 16 December 1981 using the IR photometer on the 3.9-m Anglo-Australian telescope. Photometry at J (1.20 μ m), H (1.64 μ m), K (2.2 μ m) and L' (3.8 μ m) was obtained through 5 and 10 arc s circular apertures. In all cases the galaxies were centred in the beam by maximizing the 1.2 μ m signal. A chopper throw of 12 arc s in a north-south direction was used.

The results of the photometry are given in Table 1, together

Table 1 Observed *K* magnitudes and IR colours of the four active ellipticals

IC5063	<i>K</i>	<i>J</i> - <i>H</i>	<i>H</i> - <i>K</i>	<i>K</i> - <i>L'</i>
5 arcs	11.32 ± 0.03	0.92 ± 0.04	0.48 ± 0.04	2.15 ± 0.05
10	10.58 ± 0.03	0.85 ± 0.04	0.40 ± 0.04	1.66 ± 0.08
5-10	11.34 ± 0.04	0.80 ± 0.06	0.31 ± 0.06	0.72 ± 0.09
NGC1052				
5 arcs	10.12 ± 0.03	0.77 ± 0.04	0.35 ± 0.04	0.70 ± 0.04
10	9.30 ± 0.03	0.78 ± 0.04	0.32 ± 0.04	0.57 ± 0.08
5-10	9.98 ± 0.04	0.79 ± 0.06	0.29 ± 0.06	0.23 ± 0.09
PKS2158 - 380				
5 arcs	12.33 ± 0.03	0.76 ± 0.04	0.39 ± 0.04	≤ 0.8 (2σ)
10	11.71 ± 0.03	0.73 ± 0.04	0.36 ± 0.04	≤ 0.7 (2σ)
5-10	12.61 ± 0.04	0.70 ± 0.06	0.32 ± 0.06	
PKS0349 - 27				
5 arcs	14.14 ± 0.03	0.76 ± 0.07	0.46 ± 0.04	
10	13.30 ± 0.03	0.73 ± 0.04	0.52 ± 0.04	
5-10	13.97 ± 0.04	0.71 ± 0.08	0.49 ± 0.06	
Normal elliptical		0.75	0.22	~0.3

with the calculated magnitudes and colours within the 5-10 arc s annulus surrounding the nucleus. The colours (on the AAO-Johnson system¹⁴) of normal elliptical galaxies¹⁵ are given for comparison.

Clearly the colours of PKS2158 - 380 and PKS0349 - 27 are very similar to those of normal elliptical galaxies, whereas the results on NGC1052 show the excess IR radiation in the nucleus which has been reported previously⁷.

However, IC5063 shows a very much larger (*K* - *L'*) colour of 2.1 mag within the 5 arc s aperture, while its (*J* - *H*) and (*H* - *K*) colours are close to those of normal ellipticals. This indicates the presence of a very red source which begins to dominate the light of the galaxy only at wavelengths longer than 3 μm. Furthermore, the colours measured for the 5-10 arc s annulus do not show the large excess, implying that it predominantly originates within the 5 arc s aperture. The (*K* - *L'*) colour of the annulus is slightly larger than those of normal ellipticals, but a combination of aperture misplacement and seeing could cause a slight spillage of light from the nucleus outside our 5 arc s aperture, so we regard these data as being consistent with a compact source for the IR excess.

We have used two methods to attempt to separate the colours of the IR source from the stellar component of IC5063. First, because the IR colour gradients in normal elliptical galaxies are small¹⁵, we can assume that the ratio of the flux from the stellar component in the 5 arc s aperture and the 5-10 arc s annulus is the same at all colours. Assuming the contribution of the nucleus is negligible at *J*, we can then estimate the stellar contribution within the 5 arc s aperture for each colour (see Table 2).

The second method is shown in Fig. 1, which is a plot of the observed colours in the form of a flux ratio diagram^{4,16}. On this diagram the observed colours must lie on a straight line joining the colours of the component sources. The colour of the nucleus must therefore be on the line through the 5 and 10 arc s points. A further assumption about the nature of the sources spectrum is needed to define its colours uniquely. The two natural choices for the nuclear spectrum are, non-thermal radiation represented by a power law $F_{\nu} \propto \nu^{-\alpha}$, and re-radiation by hot dust in the form of a black-body spectrum; although a range of dust temperatures can mimic a power law. For a true power law spectrum the intersection of the line with the locus of possible power law spectral indices, α , indicates a value for α of ~-4.5, whereas in the case of a black body, by a similar procedure we find that its colour temperature is ~650 K. If the IR excess is non-thermal in origin this analysis shows that the nuclear source has an extremely steep spectrum, similar to those observed in NGC1052 and the red QSOs. The spectrum of NGC1052 is much shallower, $\alpha = 1.7$ longward of 3 μm, but must steepen to at least $\alpha = -2.7$ below 2.2 μm to account for its weakness in the optical⁸. The spectra of the red QSOs also start shallower

than observed in IC5063 but again steepen rapidly to $\alpha \sim -3$ below 5 μm (ref. 17). Observation of these objects^{1,2,18} and brighter flat spectrum sources¹⁹ have shown that they possess many of the characteristics of BL Lac objects. The detection of appreciable linear polarization in 1413 + 135 (ref. 1) strongly supports their identification as steep spectrum BL Lac objects and the rapid steepening of their spectra has been convincingly explained as a sudden high energy cutoff in the distribution of synchrotron emitting electrons^{1,2}. If the luminosity range of the red QSOs approaches that of normal BL Lac objects then we might expect to find low luminosity counterparts to Mkn 421 in nearby elliptical galaxies. The nuclear IR source in IC5063 may well be just such an object.

The alternative possibility of emission from hot dust arises because the Wein tail of a black body curve can readily mimic a steep spectral index source. This explanation has proved very attractive for the IR continua of Seyfert galaxies as it avoids the physical problems associated with producing the observed spectral changes with non-thermal spectra^{20,21}. Inspection of the SRC Southern Sky Survey shows there is dust in IC5063 which could be heated by the intense UV source invoked as the origin of the photoionization of the gas. However, the high excitation of the gas at large distances from the nucleus argues that the UV source cannot be obscured as heavily as a typical Seyfert nucleus. With the limited range of our data we cannot rule out either option directly although we find the need for an alternative mechanism is capable of producing steep spectrum sources far less compelling since the discovery of the red QSOs. Observations of variations in the near IR with time scales of the order of a day would indicate the presence of a non-thermal source. Another test to distinguish between the

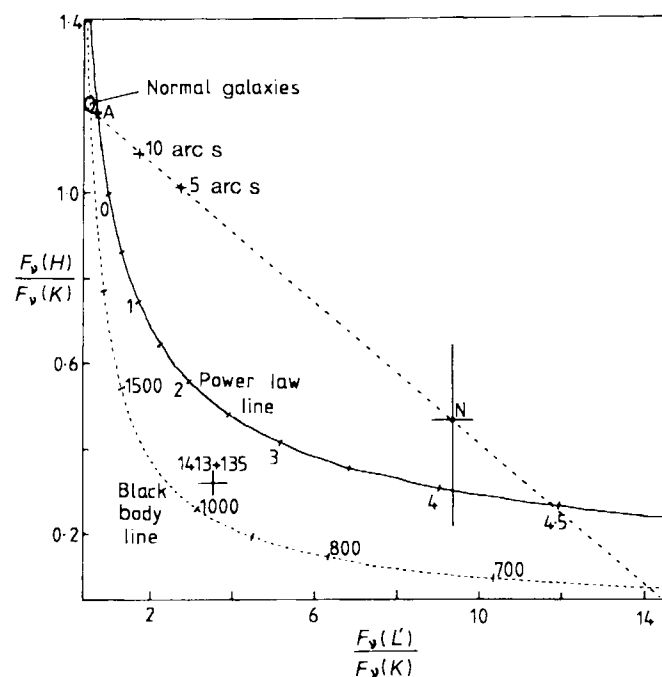


Fig. 1 Flux ratio diagram plotting the ratio of flux at *H* (1.64 μm) to *K* (2.19 μm) against ratio of fluxes at *L'* (3.8 μm) to *K* (2.19 μm). On this diagram, the point representing a composite object must lie on the straight line joining the colours of the two component sources. We have plotted the observed points for IC5063 in 5 and 10 arc s apertures and in the 5-10 arc s annulus (marked A). These points represent varying contributions from the elliptical galaxy and the central IR source. The line through these points passes through the region of the normal elliptical galaxies when extrapolated to the left. The colours of the nucleus must lie on the same line extrapolated to the right. Thus if the source is a power law it must have $\alpha \sim -4.5$. If it is a black body it must have $T \sim 650$ K. The colours deduced for the nucleus given in Table 2 are also marked (N). The approximate position of the very red QSO 1413 + 135 is marked. This source lies below the power law line as its spectrum is becoming less steep by 3.5 μm.

Table 2 IR colours of the stellar and nuclear components of IC5063

	5–10 arc s annulus	5 arc s aperture	Mag. of galaxy inside 5 arc s (assumed)	Mag of central IR source
<i>J</i>	12.45	12.72	12.72	—
<i>H</i>	11.65	11.80	11.92	14.3
<i>K</i>	11.34	11.32	11.61	12.89
<i>L'</i>	10.62	9.17	10.89	9.41

two alternatives in IC5063 would be IR polarimetry. The presence of significant linear polarization in the IR would firmly establish the non-thermal origin of the continuum. It is also important to determine the shape of the spectrum at longer wavelengths to search for either evidence of re-radiation by warm dust or the flattening which characterizes the continuum of BL Lac objects. If the IR source is non-thermal in origin then its spectrum must flatten dramatically towards the UV if it is to be responsible for photoionizing the gas. We should also stress that our observations do not preclude the existence of bright non-thermal nuclei in PKS2158–380 and PKS0349–27 provided their spectra steepen at longer wavelengths than in IC5063.

Similar observations for a complete sample of radio ellipticals would establish the frequency of this type of nucleus.

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The source of Saturn electrostatic discharges

D. R. Evans*, J. H. Romig*, C. W. Hord†, K. E. Simmons‡, J. W. Warwick* & A. L. Lane‡

* Radiophysics Inc., 5475 Western Avenue, Boulder, Colorado 80301, USA

† Laboratory for Atmospheric and Space Physics, University of Colorado, Boulder, Colorado 80309, USA

‡ Jet Propulsion Laboratory, California Institute of Technology, Pasadena, California 91109, USA

During both Voyager encounters with the saturnian system, the Planetary Radio Astronomy (PRA) experiment detected strong discrete episodic bursts of radio emission, termed Saturn electrostatic discharges (SED)^{1–3}. An examination of Voyager 2 photopolarimeter (PPS) data now reveals a narrow feature (possibly a gap) in Saturn's B ring. A single, unique object appears to be responsible for both the SED and this feature.

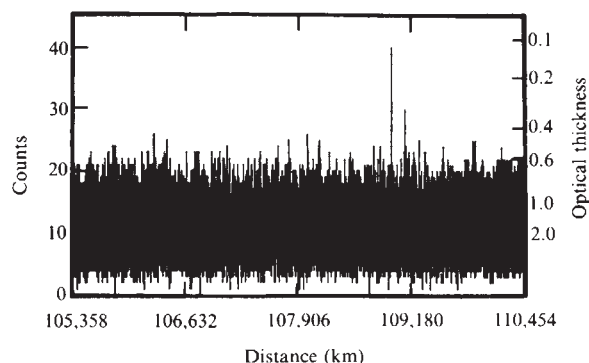


Fig. 1 Optical thickness of 5,000 km of the outer B ring at 2,650 Å (300 Å bandwidth), as determined by the Voyager 2 photopolarimeter during the Delta Scorpis occultation. The optical thickness is calculated from the number of counts received at the instrument in a given 10-ms sample time.

The episodic nature of SED was well defined at both Voyager 1 and Voyager 2 encounters, the mean period being 10 h 10 ± 5 min and 10 h 11 ± 5 min, respectively, after spacecraft motion was taken into account. The simplest, but by no means only, explanation of this episodic behaviour is that the SED source is an object in a circular, equatorial orbit 107,990–109,200 km from the centre of Saturn. For this hypothetical object to be the source of SED, it must be distinct from its environment by virtue of physical, chemical and/or electrical properties. Such an object should have an effect on surrounding ring material. Indeed, both the Voyager 2 high-resolution (100 m) photopolarimeter and the lower-resolution (~5 km) imaging aboard both spacecraft show remarkable features in this region of the rings.

The PPS occultation data, which are taken at a wavelength of 2,650 Å, show a relatively opaque region from 105,000 to 110,000 km within the B ring (Fig. 1). This region has a mean optical depth in excess of 1.8; however, embedded within this part of the B ring there is a single feature ~150 m wide in which the apparent optical thickness is much less than this value (Fig. 2). This feature is located at $108,942 \pm 6$ km [based on a best fit to experimental values of the Saturn pole position by J. Holberg (personal communication)]. The apparent optical depth in the middle of the feature is 0.15. Like all experimental measurements, this number has an associated error; we calculate that the true optical depth at this point is less than 0.50, with 99.5% certainty. In fact, due to the finite instrument response time and thickness of the ring at this point, these numbers are very much upper limits; the data are certainly not inconsistent with a narrow region of much lower optical depth. To ascertain the reality of this feature, as opposed to the possibility of a statistical fluctuation occurring in the PPS instrument, we have plotted the frequency distribution of the PPS counting rate for 48,000 points in the vicinity of the feature (Fig. 3). The number of counts recorded by the PPS instrument in a given 10-ms sample is proportional to the number of photons received at the instrument in that time. The recorded number of counts is thus related to optical thickness in a logarithmic manner.

The frequency distribution displayed in Fig. 3 has a mean of 11.745 counts and a standard deviation of 3.2935 counts. For comparison, Fig. 3 also shows a gaussian curve with these parameters. Generally, the two curves agree closely, except in the wings. Using this gaussian as a model, we can at least estimate the probability that the feature is produced by statistical fluctuation, within an order of magnitude or so. The feature comprises two adjacent points, with readings of 40 and 25 counts. The combined probability of obtaining two such adjacent points is of the order of 10^{-22} . We have nearly 48,000 possible adjacent pairs, so the probability of obtaining the

observed result by chance somewhere in the data is about 10^{-17} ; even allowing for the lack of accuracy in the model gaussian, this result clearly shows that the feature is almost certainly not statistical in origin. In addition, we have examined over 100,000 independent data points taken by the PPS instrument in its occultation mode at other times, and we find no trace of a similar event anywhere in this enormous data set.

A second peak, of 30 counts, is also visible in Fig. 1. Applying similar statistics, we obtain a probability of 5×10^{-4} of chance occurrence. However, in this instance, only a single data point is significantly different from background values, and, as we have already noted, the gaussian model does not fit the data exactly in the wings, so we feel that this second feature may well be statistical in origin.

The PPS occultation data therefore indicate that at a radial distance of $\sim 108,942$ km there is a region of lowered optical thickness some 150 m wide. As our best estimate of the optical thickness in the middle of the feature is 0.15, we believe that it is reasonable to describe the feature by the term 'gap'.

The radial distance of $108,942 \pm 6$ km corresponds to an orbital period of $10 \text{ h } 9 \text{ min } 4 \text{ s} \pm 3 \text{ s}$ (J. Anderson, personal communication), after planetary oblateness is taken into account. This level of accuracy of period determination permits us to combine unambiguously the Voyager 1 and Voyager 2 SED databases, thus providing an effective baseline of some 290 days from which to estimate the period of the SED source. The PRA data alone provide us with a 'comb' of possible periods with 'tooth' separation of $\sim 54 \text{ s}$. If we assume that the SED source may be uniquely identified with the PPS gap, the PPS data should permit us to identify a particular 'tooth' which is consistent with both experimental results. We have indeed found that the data identify a tooth with a period of $10 \text{ h } 9 \text{ min } 12 \text{ s} \pm 6 \text{ s}$, which is consistent with the orbital period above. It is reassuring that the two different sets of data are consistent with a single period, although this fact cannot be described as statistically significant, as the chance probability of this being possible is quite high— $12/54$, or $\sim 22\%$. Bearing in mind our previous argument concerning the uniqueness of the SED source, we believe that it is highly likely that there is a single cause of both SED and the PPS occultation gap observation.

In addition to the PPS data, Voyager images of this region show two intense azimuthal bands which appear bright in forward scattered light, indicating that they comprise a local enhancement of the micrometre-sized dust population, which is ubiquitous throughout the outer two-thirds of the B ring. These bands are each roughly 300 km wide and are $\sim 2,000$ km apart. Although it is true that unusual features are to be seen on Voyager images throughout this region, these two bands are

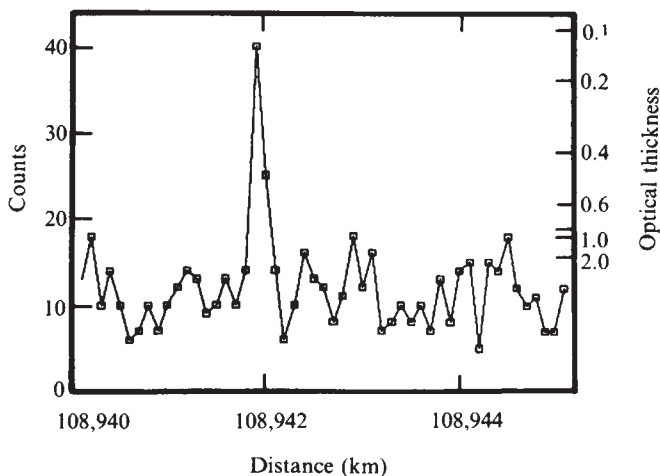


Fig. 2 Detailed structure of the B ring near the large spike in Fig. 1; this structure indicates a narrow (~ 150 m) gap of low optical thickness.

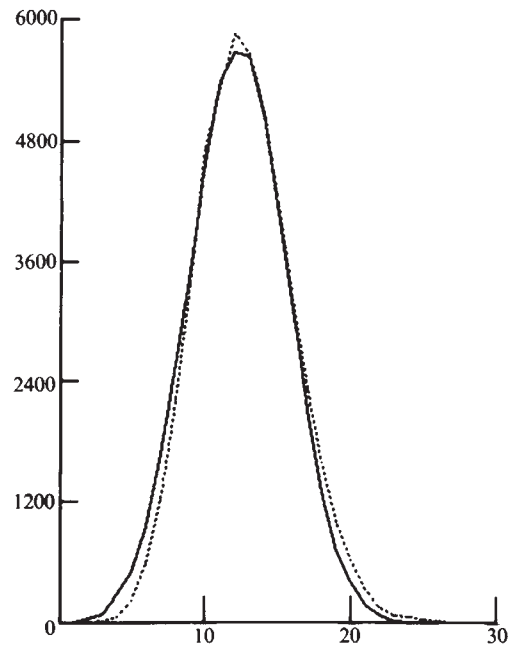


Fig. 3 Occurrence frequency versus counts for 48,000 data points obtained in the region shown in Fig. 1. Experimental data are shown dotted. The solid line is a gaussian with mean 11.745, s.d. 3.2935.

the most prominent features in wide-angle images of this part of the ring system. Furthermore, enhanced colour images of the ring system indicate a change in particle population characteristics at or near the region in question. This change, and indeed the very existence of micrometre-sized dust in the outer B ring, may be a direct result of the presence of the object and/or the associated SED.

Voyager 2 also carried a UV spectrometer which was boresighted with the PPS instrument and simultaneously observed Delta Scorpii in the range $900\text{--}1,700 \text{ \AA}$. This spectrometer failed to detect any reduction in optical depth at the PPS gap location (with 99.9% confidence) (J. Holberg, personal communication). It is possible that the 'gap' is in fact populated with small Rayleigh scatterers (of the order of $\leq 100 \text{ \AA}$ in diameter; several theories indicate that there may be substantial numbers of such scatterers in the rings—see, for example, ref. 4). Whether or not this is the true reason for the UV spectrometer null result, the observation that the optically dense outer B ring seems capable of sustaining a very narrow gap (at least to $2,650 \text{ \AA}$ radiation) is important in developing a theoretical understanding of the ring.

Thus, Voyager PRA and PPS data indicate the presence of a single, unique object in the B ring at a distance of $\sim 108,942$ km from the centre of Saturn. This object exerts an influence on material adjacent to its orbit, resulting in the formation of a 150-m gap along that orbit. In addition, Voyager imaging data suggest that the object may influence micrometre-sized dust hundreds of kilometres away. The interaction between this object and its environment seems to be the source of SED.

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STARE observations of long discrete echoes

E. Nielsen*, G. Sofko† & W. I. Axford*

*Max-Planck-Institut für Aeronomie, D-3411 Katlenburg-Lindau 3, FRG

†University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0

Early studies of auroral radar echoes by Unwin^{1,2} showed the existence of a particular class of 'long discrete' echoes, distinguished as being weak and relatively long-lasting (1–60 min). The backscatter usually consists of several narrow (~20 km) bands which drift slowly in latitude, and the height of the backscattering region appears not to exceed 100 km. Here, we report on observations of this type of radar aurora made during the past few years with the Scandinavian Twin Auroral Radar Experiment (STARE). Whereas Unwin's observations covered the range of geomagnetic latitudes corresponding to I -values ~3–5, the present study is concerned with higher latitudes, corresponding to the range I ~5.7–8.

The STARE system³ provides measurements of the backscatter intensity and irregularity drift velocity over a large area (~200,000 km²) with good spatial resolution (20 × 20 km²) and a temporal resolution of ~20 s. The twin radar system permits Doppler velocities of E-region ionospheric irregularities to be measured stereoscopically. Aliasing around the time base is avoided by the use of a low pulse repetition frequency (0.05 Hz). Measurements from a given volume of space are combined to yield the irregularity drift velocity vector, assuming that the Doppler velocity measured in a given direction equals the component of the irregularity velocity in that direction. The operating frequencies of the two radars are ~140 MHz, so that they are sensitive to irregularities with wavelengths of the order of 1 m. The system has an automatic gain control which is adjusted to the strongest backscatter detected; thus regions of relatively weak scatter cannot be detected in the presence elsewhere of regions of strong signal-to-noise ratio, which, of course, can effect the apparent probability of occurrence of the former.

The characteristics of the long discrete auroral radar echoes, as observed by STARE, are as follows: (1) On a range-time-intensity plot (Fig. 1a) the echoes appear as a series of striations

which may persist for an hour or more (time interval II in Fig. 1a). (2) The striations are rather straight and are narrow in range (~20–100 km) in contrast with the spread, diffuse appearance of the more intense backscatter associated with strong auroral activity (time interval I in Fig. 1a). (3) The backscatter intensities are weak, with a typical signal-to-noise ratio of only a few decibels. (4) The speed of the irregularities as obtained from Doppler measurements (0–400 m s⁻¹) and the speeds of the apparent motions in range of the backscattering regions (100–300 m s⁻¹) are much smaller than the speeds usually associated with radar aurora (~300–3,000 m s⁻¹). (5) The echoes commonly occur following periods of intense radar auroral activity associated with magnetic disturbances, as indicated in the Fig. 1. (6) The echoes appear to be most prevalent in the early morning and late afternoon hours and in winter rather than summer.

The clear distinction between the properties of intense radar aurora occurring during disturbed periods and the long discrete echoes is evident in Fig. 1b, c where the distributions of the signal-to-noise ratio (SNR) and Doppler-determined velocity (speed and direction) are shown for the intervals I and II. It is evident from Fig. 1b that the backscatter intensity of discrete echoes (average SNR ~5 dB) is in general much less than that from ordinary radar aurora (average SNR ~18 dB). As emphasized above, discrete echoes might also be present in interval I but they would be masked in the presence of the more intense and diffuse radar aurora associated with very active periods and as a result of the automatic gain control of the STARE receivers.

There is a very clear difference in the speed distributions between intervals I and II, shown in Fig. 1c. During the strongly disturbed period, which includes interval I, the observed speed distribution shows an almost complete cut-off at speeds below 300 m s⁻¹, whereas in the period dominated by discrete echoes the average speed is only 150 m s⁻¹ and hardly ever exceeds 300 m s⁻¹. It is interesting that during interval II in Fig. 1 the speed of the polewards motions of the backscatter striations is comparable with but not exactly the same as those obtained from the simultaneous Doppler measurements. The angular distribution of the Doppler velocities measured during the two intervals is shown in Fig. 1c; in interval I the directions lie in the sector 0–90° east of north with a maximum at ~75°; in contrast, the angular distribution during interval II is much broader, being predominantly northwards but with deviations of 70° and more, both east and west of north.

The diurnal and seasonal distributions of the occurrence of discrete echoes observed during 5 yr between 1977 and 1981 are shown in Fig. 2. It is clearly evident that this type of radar

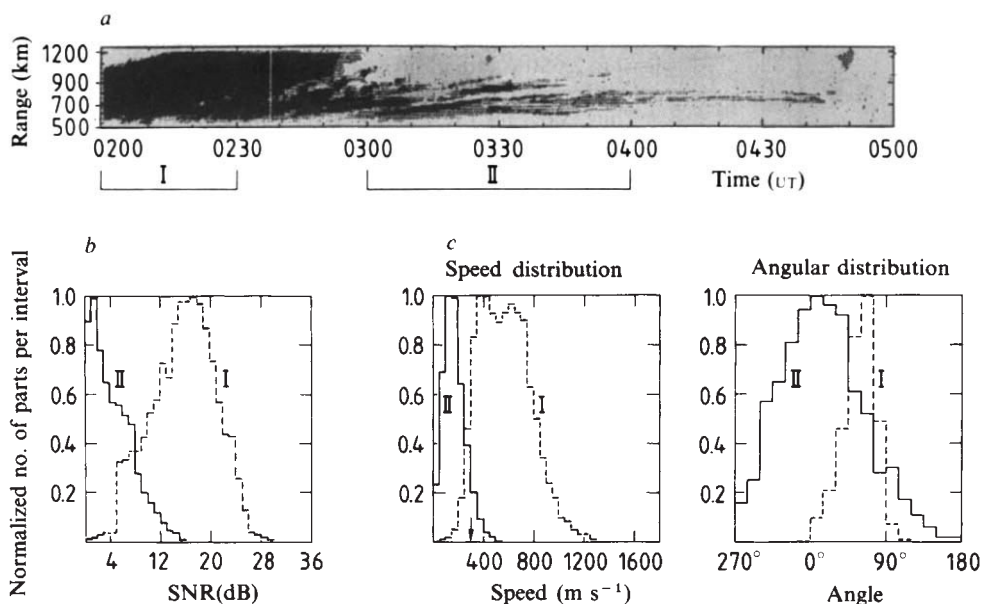


Fig. 1 a, A range-time-intensity plot showing ordinary radar aurora and long discrete echoes detected by a single narrow antenna beam pointing 24.5° E of geographic north (beam B). The signal in time interval I is representative of the radar aurora associated with auroral electrojet activity. The striated appearance of the record in interval II indicates the presence of long discrete echoes. b, Statistical distributions of the signal-to-noise ratios measured during intervals I and II. The long discrete echoes are significantly weaker than the ordinary radar aurora. c, Distributions of the irregularity drift speeds and directions in intervals I and II. The long discrete echoes have drift speeds which are less than the threshold of the two-stream instability (arrow).

aurora is almost entirely confined to periods of EUV 'darkness' at E-region altitudes and is predominantly an early morning phenomenon. Note, however, that the presence of intense backscatter would tend to mask the presence of any discrete echoes during the period 2000–0200 h and hence there is a possible bias in the diurnal distribution.

The most remarkable feature of the discrete echoes is the speed distribution. It is usually assumed, on the basis of linear instability theories of plasma processes occurring in the auroral ionosphere, that the Doppler velocities determined by STARE measurements are essentially the same as the electron drift velocities and hence provide a measurement of the E-region electric field^{4–6}. The two-stream instability⁷ is the most likely candidate for producing the density irregularities observed to occur in regions of strong auroral zone electric currents such as observed during interval I, for example. However, this instability has a threshold such that the electron drift speed relative to the ions must exceed the ion sound speed, which is typically 300 m s^{-1} (equivalent to $\sim 15 \text{ mV m}^{-1}$). The speed distribution observed during the period of occurrence of discrete echoes shown in Fig. 1c, is clear evidence that the electron density irregularities cannot be due to the two-stream instability. Because we do not know the nature of the electron density irregularities we cannot assert that the measured apparent speeds represent electron drift speeds and hence they may not necessarily constitute a measurement of the ionospheric electric field strength.

It is possible that the electron density irregularities associated with striated radar aurora are due to an instability other than the two-stream instability, for example the gradient-drift instability⁸. However, to produce this instability with an eastwards directed electric field a strong eastwards directed electron density gradient is required. This is difficult to accept in view of the fact that the striations are evidently narrow in north-south extent and persist for upwards of an hour. The length scales involved ($\sim 20 \text{ km}$) do not in any case suggest that the electron density gradient is large.

It is difficult to argue that the electron density irregularities which produce the discrete echoes observed by STARE are associated with magnetospheric effects. A mapping to ionospheric levels of small-scale magnetospheric motions (or, equivalently, electric fields) must be ruled out as a possible cause of the irregularities because for such small-scale lengths perpendicular to the magnetic field, as required in this case, the mapping is quite ineffective⁹. It is also unlikely that the source of the ionization produces electron density irregularities of the required scale directly, as the gyroradii of electrons with energies of the order of 10 keV (necessary to penetrate to altitudes of $\sim 100 \text{ km}$) are $\sim 10 \text{ m}$, which is much larger than the irregularity scale size required. It is also conceivable, however, that a combination of a strongly inhomogeneous source distribution and the gradient drift instability, together with some cascading in irregularity scale could be effective¹⁰.

If the discrete echoes are associated with enhanced electron precipitation it is conceivable that the irregularities are due to unstable electron velocity distributions resulting from a relatively high density of secondaries. If the secondary electron distribution function is indeed unstable, such irregularities should be present in all regions of intense electron precipitation, especially around arcs; in general an anticorrelation of backscattering and precipitation regions is observed (G. Keys and M. Scourfield, personal communication), although this could be affected by the automatic gain control in the STARE system. Such an explanation would be consistent with the slow, polewards drift observed in the early morning post-substorm aurora¹¹; however it is not obvious that field-aligned irregularities can be produced at relatively low altitudes by this means.

In view of the fact that both the magnetospheric and ionospheric explanations for the origin of the irregularities producing the discrete echoes are not completely satisfactory, it is interesting to consider whether or not an atmospheric origin is a

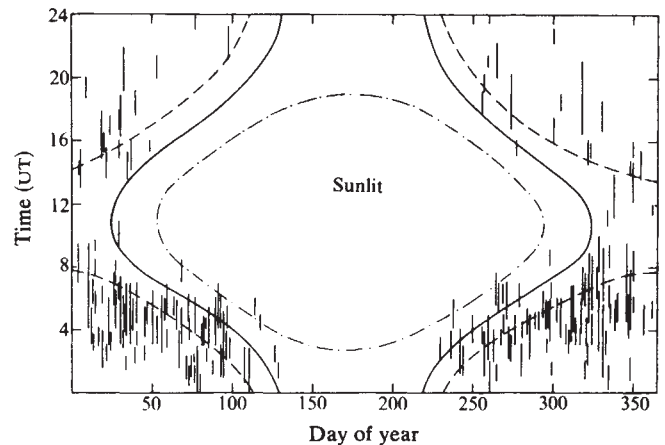


Fig. 2 The diurnal and seasonal occurrence of long discrete echoes. Contours of constant solar elevation ($0^\circ \pm 10^\circ$) in the middle of the STARE field of view are shown and indicate that the discrete echoes are not present, within the limits of the system sensitivity, for solar elevation angles $\geq 10^\circ$. ---, -10° ; —, 0° ; - - - - , 10° .

possibility. In this case, the echoes would be similar to those observed by mesosphere-stratosphere-troposphere radars such as the SOUSY system¹². The electron density irregularities are then most likely to be the result of an interaction between turbulent motions of the neutral gas and steep electron density gradients, although other processes such as density fluctuations associated with the turbulence itself, which produce corresponding fluctuations in the electron density (by entrainment or as a result of the ionizing processes), are also possibilities^{13–15}. In this case however, the backscatter must occur at much lower altitudes than usually assumed to be the case for radar aurora, as the STARE scattering scale length of $\sim 1 \text{ m}$ is equal to the mean free path in the neutral atmosphere at an altitude of $\sim 112 \text{ km}$. To produce a reasonable level of fluctuations in the neutral density the mean free path should be $\ll 1 \text{ m}$, which suggests that the scatter should originate at altitudes $\leq 100 \text{ km}$, and even then the length scale would lie in the viscous subrange of the atmospheric turbulence. This explanation has certain attractive features, notably that it does not require a velocity threshold and, indeed, the observed speeds are comparable with the speeds of atmospheric motions. To test this hypothesis it will be necessary to determine more accurately the height of the region from which the echoes come, which, according to Unwin^{1,2}, is '100 km or less'.

The disappearance of E-region irregularities after sunrise could be explained as a consequence of an enhanced recombination rate. It can be shown that the decay time of an irregularity is $1/2\alpha\bar{n}$, where α is the (dissociative) recombination coefficient and $\bar{n}$ the mean electron density. As $\bar{n}$ may increase by a factor of 10 or more after sunrise, it is clear that the effects of any source of electron density fluctuations must be strongly suppressed in such circumstances. However, if the irregularities occur at much lower (D-region) altitudes, this argument is not necessarily correct, because photodissociation of negative ions may have an important role during sunrise. In this case the disappearance of the echoes would be more easily accounted for on the basis of a complete change in the electron density profile as well as a possibly enhanced loss rate.

To make further progress in understanding the phenomenon of discrete radar echoes in the auroral zone, further observations are required. For example, it will be necessary to determine whether or not striations as shown in Fig. 1 correspond to regions of enhanced optical emission and enhanced ionospheric plasma density. The latter can be determined using the EISCAT system, now being brought into operation, and this will also allow us to determine whether or not the Doppler velocities measured by STARE are related to electric field drifts. More extensive latitude coverage will also be required,

for example, to determine the relationship of the discrete echoes to phenomena occurring near the auroral electrojet. This is now possible using STARE together with the new SABRE system, which is similar to STARE but operates in a lower latitude range ($4.2 \leq l \leq 5.6$).

Finally, direct measurements of the altitude of the echo-producing irregularities should be possible by using the partial reflection system existing at the EISCAT site and other backscatter radars which are available, including the EISCAT VHF system.

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Constancy of oceanic deposition of ^{10}Be as recorded in manganese crusts

T. L. Ku & M. Kusakabe

Department of Geological Sciences, University of Southern California, Los Angeles, California 90089-0741, USA

D. E. Nelson, J. R. Southon, R. G. Korteling, J. Vogel & I. Nowikow

Archaeology and Chemistry Departments, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6

Recent developments in the use of nuclear accelerators as high-energy mass spectrometers^{1,2} has renewed interest in the study of cosmogenic ^{10}Be (half life 1.5 Myr) as a geophysical time tracer. The accelerator techniques enable us to measure ^{10}Be with a sensitivity of 10^7 atoms or less. The potential age dating span with ^{10}Be is extended back to at least 15 Myr BP, providing that the ^{10}Be production and deposition rates remain constant or nearly constant over that time period. A knowledge of the long-term constancy in the ^{10}Be deposition rate is thus very important. We report here the first detailed measurements of ^{10}Be and ^9Be in two ferromanganese oxide crusts from the sea floor of the equatorial Atlantic (sample no. K-9-21) and the North Pacific (SCHW-1D), each representing >10 Myr of accumulation history. We have found in these crusts that the deposition of ^{10}Be and $^{10}\text{Be}/^9\text{Be}$ during the past 7–9 Myr has been constant. Averaged over time intervals of ~1 Myr, the variation is within $\pm 6\%$. Before that time, both crusts show similar significant deviations. The 7–9 Myr demarcation may be related to the reported late Miocene global abyssal circulation change in the ocean³.

The extent of variation in the oceanic deposition rate of ^{10}Be during the past 2.5 Myr has been assessed based on the ^{10}Be distribution in sediment cores^{4,5}. An upper limit of $\pm 10\%$ has been placed on the variation when the deposition is integrated over time periods of $2\text{--}7 \times 10^5$ yr. For shorter periods of 1×10^5 yr (roughly corresponding to climatic cycles), the limit is placed⁵ at $\pm 30\%$. The record can be extended by examining ^{10}Be distribution in deep-sea ferromanganese deposits as these materials, in addition to being relatively free of terrestrial detrital input, contain very long depositional records due to their slow accretion rates⁶. Manganese crust K-9-21 was

dredged from a fracture zone scarp on the Sierra Leone Rise. It rested on an altered foraminifera ooze. The substrate of SCHW-1D was a large basaltic rock. These crusts were chosen over the more commonly occurring manganese nodules for the study because the crusts were: (1) relatively flat, so that sampling of contiguous layers perpendicular to the growth axis could be accurately done; (2) fixed in their growth position with respect to top and bottom orientations relative to the sea floor.

Thin layers parallel to the growth surface were carefully taken throughout the whole thickness of the crusts for analyses of ^{10}Be , ^9Be , Mn, and Fe. The thickness of each layer was estimated using caliper and/or from the area, weight and density of material scraped off⁷. In the accelerator analyses of ^{10}Be , milligramme-to-gramme-sized samples were leached with hot 8 M HCl, and then to the leachate was added a $^9\text{BeSO}_4$ carrier (equivalent to 1–10 mg BeO). A simplified version of the procedures of Ku *et al.*⁸ involving only the ion exchange steps was used in the separation of Be from the leachate, as strict chemical purity is not required by the accelerator technique. Measurements of $^{10}\text{Be}/^9\text{Be}$ were carried out using the McMaster University tandem Van de Graaff accelerator modified for this purpose⁹. The sensitivity achieved was $\sim 5 \times 10^{-13}$, and background intensities from reagent blank runs were <10% of the weakest sample signals measured. Mn, Fe and total Be were measured by atomic absorption spectrophotometry; to measure Be, flameless atomization with a deuterium background corrector was used.

The analytical results are presented in Table 1, with errors shown as $\pm 1\sigma$. For Mn and Fe, the measurement precisions are about $\pm 1\%$. The concentration values are listed on a residue-free basis, as the Mn, Fe, and Be will be mainly associated with the HCl-leachable oxide phase of the crusts²⁴.

The temporal variation of ^{10}Be deposition can be assessed by examining the depth distribution of ^{10}Be and the other chemical constituents. As the major constituents in ferromanganese deposits, Mn and Fe are often anti-correlated, and their ratios can be used as a measure of chemical and growth rate variability^{10,11}. In this regard, the two samples studied both show narrow limits of variability. As seen in Table 1, concentrations of Mn and Fe in the two specimens are comparable and show variations of about $\pm 10\%$, with Mn/Fe ratios ranging only from 0.7 to 1.0. Except for the low values in surface layers of SCHW-1D, ^9Be also shows minor temporal fluctuations of about $\pm 10\%$. These narrow limits of variation suggest overall uniformity in the deposition of the two crusts. The log-linear distribution of ^{10}Be shown in Fig. 1 supports this uniformity: the straight lines are weighted least-squares fits to an exponential function, as obtained through iterations¹². The effective measurement depth for each sample is calculated iteratively to account for the radioactive decay taking place during the deposition period represented by each sample interval. The fittings are made over the 'linear' segments of the plots (see legend to Fig. 1), covering major portions of both samples. The slopes of the straight-line fits, which yield accretion rates for the crusts, have standard errors of $\pm (4\text{--}7)\%$ (averaging $\pm 5.5\%$). These errors denote the scatter of data about an exponential curve, and therefore limits the constancy of the ^{10}Be flux and of the flux ratio ^{10}Be to ^9Be into the deposits.

Both the growth rate (~ 1.5 mm Myr) and the Mn/Fe ratio (< 1) are near the lower end of their reported ranges of values for Mn nodules^{6,11}, suggesting minimum influence of diagenesis as a source for Mn. The crusts have received $\sim 2 \times 10^4$ atoms $\text{cm}^{-2} \text{yr}^{-1}$ of ^{10}Be , which is $\sim 4\%$ of the global average ^{10}Be production rate^{13,14}. A similar situation applies to the Mn inventory: ~ 0.05 mg $\text{cm}^{-2} \text{Kyr}^{-1}$ in the crusts compared with 1.3 mg $\text{cm}^{-2} \text{Kyr}^{-1}$ as the average Mn flux into 38 deep-sea cores¹⁵. Thus most of the ^{10}Be and Mn must have deposited in the adjacent sediment, or the accretion process is not continuous; that is on average, the crusts were actually growing for $\sim 4\%$ of their lifetime^{8,16}.

A decrease of ^{10}Be concentration toward the surface from a near-surface maximum occurs in SCHW-1D (Fig. 1). The

Table 1 Analytical data on Mn crusts

Depth in crust (mm)	HCl insoluble residue (%)	Mn (%)	Fe (%)	⁹ Be (p.p.m.)	¹⁰ Be ($\times 10^8$ atoms g ⁻¹)	¹⁰ Be/ ⁹ Be atom ratio (= 10^{-9})
K-9-21 (7°58'N, 21°02'W; 1,800 m)						
0-1.5	2.1	16.8	22.3	9.89 ± 0.22	488 ± 49	74.0 ± 7.6
1.5-3	2.8	17.8	25.4	10.4 ± 0.7	387 ± 28	55.6 ± 5.5
5-7	7.4	19.2	26.8	13.2 ± 0.5*	107 ± 11	12.2 ± 1.3
10-13	4.9	19.6	24.9	12.3 ± 0.8	15.7 ± 1.6	1.85 ± 0.23
13-17	2.7	17.3	18.3	9.23 ± 0.92	10.3 ± 0.6	1.66 ± 0.19
17-23	4.0	17.6	21.5	9.88 ± 0.99	2.95 ± 0.58	0.45 ± 0.10
Weighted average	4.0	18.0	22.3	10.6	—	—
SCHW-1D (30°N, 140°W; 3,840 m)						
0.00-0.09	29.5	20.1	23.7	3.86 ± 0.35	479 ± 23	186 ± 19
0.09-0.16	25.2	16.7	22.7	2.91 ± 0.21	276 ± 27	142 ± 17
0.16-0.29	24.1	16.7	24.0	5.05 ± 0.21	372 ± 21	110 ± 8
0.29-0.48	18.0	16.7	22.6	4.82 ± 0.26	384 ± 17	119 ± 8
0.48-0.85	9.6	17.7	22.2	6.00 ± 0.60	377 ± 32	94.0 ± 12.3
0.85-1.66	5.2	17.0	20.1	5.87 ± 0.45	361 ± 17	91.9 ± 8.3
1.66-3.16	10.7	18.1	20.7	6.43 ± 0.50	269 ± 13	62.4 ± 5.7
3.16-4.66	15.4	20.3	24.8	8.23 ± 0.04	151 ± 5	27.5 ± 0.9
4.66-6.16	16.7	21.0	24.1	7.70 ± 0.50	116 ± 7	22.6 ± 1.4
6.16-7.66	20.2	21.8	22.3	7.69 ± 0.50	69.4 ± 2.3	13.5 ± 1.0
7.66-9.16	27.4	21.8	23.1	8.11 ± 0.24	50.1 ± 2.5	9.24 ± 0.55
9.16-10.7	22.4	21.6	21.9	7.99 ± 0.28	35.8 ± 1.6	6.70 ± 0.38
10.7-12.2	24.6	22.9	23.2	9.81 ± 0.60	31.0 ± 1.6	4.73 ± 0.38
12.2-14.7	24.2	20.7	22.2	9.10 ± 0.70	26.4 ± 1.7	4.33 ± 0.43
14.7-17.2	31.7	18.6	23.7	9.55 ± 0.45	20.4 ± 1.2	3.19 ± 0.24
Weighted average	21.2	20.2	22.7	8.1	—	—

Concentrations are reported on a residue-free basis.

* Dr C. Measures (MIT) obtained a value of 12.8 ± 0.5 p.p.m. using a gas chromatographic technique.

sampling resolution is not sufficient to detect such a feature in K-9-21. But we have found this feature to be rather ubiquitous among the other deep-sea ferromanganese deposits we have studied. This ¹⁰Be 'inversion' occurs at a depth corresponding to ~0.5 Myr in age. Whether it reflects a change in the ¹⁰Be input or a diagenetic phenomenon remains to be resolved. Noting a surface depletion of ⁹Be as well as ¹⁰Be (Table 1), two of us (M.K. and T.L.K. in preparation) have proposed a post-depositional Be fixation process whereby both Be isotopes are released from a labile phase (or phase with exchangeable Be) at the seawater-solid interface and transferred to the oxide phase through diffusion and adsorption reaction in pure fluid.

As pelagic sediment and shale contain only 2-3 p.p.m. of Be (ref. 17), most of the Be in the samples, like many other elements in ferromanganese deposits, must be authigenically precipitated rather than of detrital origin. Recent studies have described the particulate cycling of ¹⁰Be and ⁹Be in the sea¹⁸⁻²⁰. Information on the extent to which the oceanic reservoirs of these two isotopes are equilibrated in time and space is germane to the ¹⁰Be geochronology. If isotope equilibrium is achieved, the use of the ¹⁰Be/⁹Be ratio in lieu of ¹⁰Be concentration would circumvent the latter quantity's being affected by factors other than radioactive decay, such as changes in sedimentary composition and deposition rate. The present data suggest that for periods of the order of 1 Myr the oceanic ¹⁰Be/⁹Be reservoir as well as ¹⁰Be deposition has remained constant to within ±6% for approximately the past 8 Myr (represented by the first 10-13 mm of the crusts; Fig. 1).

It should also be of interest to determine the spatial constancy of the ¹⁰Be and ⁹Be reservoirs. This information would be best obtained from simultaneous measurements of ¹⁰Be and ⁹Be in water columns from various parts of the ocean. But these have yet to be done. A less rigorous alternative would be to compare reported values for the isotope ratio ¹⁰Be/⁹Be (extrapolated to zero age) in manganese nodules and crusts from different areas²¹. These values range over one order of magnitude²². To eliminate any interlaboratory discrepancies, we list in Table 2 the extrapolated ratios in six ferromanganese samples based

on measurements made only at our own laboratories, including the two crusts reported here. The data scatter is outside the experimental uncertainties of about ±15%, suggesting variability of ¹⁰Be/⁹Be with location and water mass. This is plausible, in view of the fact that the residence time of Be in the sea has been estimated¹⁸⁻²³ as being comparable with or shorter than the oceanic overturn time of ~10³ yr. If so, the temporal constancy in ¹⁰Be/⁹Be for the past 7-9 Myr deduced in this study would imply considerable stability (on a Myr time scale) in oceanic circulations during the period. However, conditions before 7-9 Myr BP could be significantly different, as shown by the deviations from the log-linear fits deeper in both crusts (Fig. 1). According to Ciesielski *et al.*³, the abyssal circulation then was very different due to the absence of a true Antarctic Bottom Water similar to that produced today in the Weddall Sea.

The Fig. 1 deviations are such that they can be explained by an approximately twofold increase in the accretion rate of the crust. Although possible, such a large increase seems unlikely considering the following. A near-synchronous accretion rate change occurring in two different major ocean basins would call for events affecting the global supply of Mn and/or Fe, such as changes in submarine hydrothermal activity and sea floor redox condition. And one would expect the samples to show a major change in their Mn/Fe ratio and perhaps ⁹Be

Table 2 Extrapolated (to zero age) atom ratio ¹⁰Be/⁹Be in six ferromanganese deposits

Sample no.	Lat., long.	¹⁰ Be/ ⁹ Be ($\times 10^{-7}$)
K-9-21	7°58'N, 21°02'W	0.96
K79-05-47BC	11°01'N, 140°03'W	3.0
Mn 139	20°01'N, 136°36'W	2.3
Aries-13D	20°45'N, 173°40'E	1.2
SCHW-1D	30°N, 140°W	1.2
Rama 1-24GC	30°N, 158°W	1.3

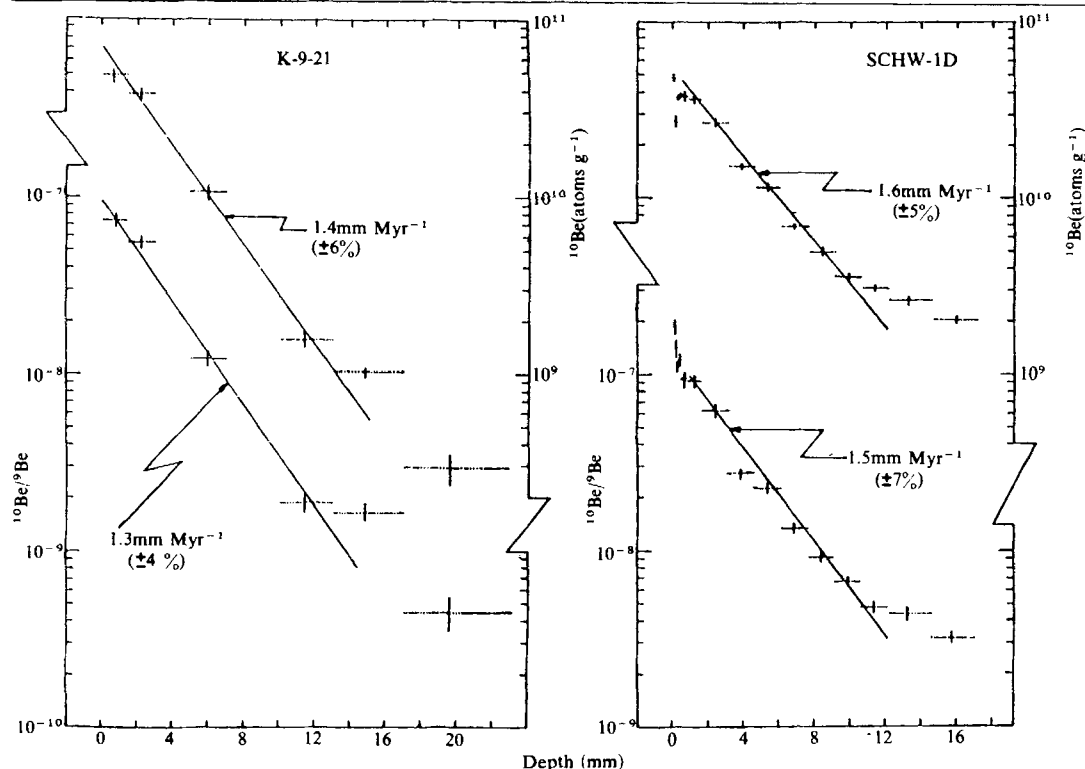


Fig. 1 Depth plots of ^{10}Be concentration (on a residue-free basis) and $^{10}\text{Be}/^9\text{Be}$ in two Mn crusts. Least squares fits for the 'linear' segments of the plots give estimates for the accretion rates (and their standard errors) of the crusts. The linear segment for sample K-9-21 includes the top four data points; for SCHW-1D, data points 6–12 from the crust surface. The horizontal dotted lines denote the depth intervals analysed, and the positions of the vertical lines (error bars) are placed at the effective measurement depths.

and residue concentrations across the 7–9 Myr boundary. We do not discern such a change. We thus favour the possibility that those deviations were caused by a shift in the initial $^{10}\text{Be}/^9\text{Be}$ ratio resulting mainly from a shift in the ^{10}Be input. While the primary atmospheric supply of ^{10}Be might have varied, we consider it more likely that the ^{10}Be input change is related to the modification of oceanic mixing patterns 7–9 Myr ago³, as mentioned above. During that time, the major production of the Antarctic Bottom Water began. This event marks the initiation of the modern global circulation, and has led to shifts in such oceanic properties as carbonate compensation level and carbon isotopic composition³. It could also have influenced the Be records observed here, by exposing the crusts to bottom waters bearing different ^{10}Be contents and $^{10}\text{Be}/^9\text{Be}$ ratios before and after the 7–9 Myr event. Studies of sedimentary deposits (such as Deep-Sea Drilling Project sediments) as well as other Mn crusts would be required to test this possibility further.

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<33,000-yr K–Ar dating of the volcano–tectonic horst of the Isle of Ischia, Gulf of Naples

P.-Y. Gillot

Centre des Faibles Radioactivités, Laboratoire mixte CNRS-CEA, 91190 Gif-sur-Yvette, France

S. Chiesa, G. Pasquare & L. Vezzoli

Istituto di Geologia, Università di Milano, sezione di Bergamo, 23100 Bergamo, Italy

The Gulf of Naples corresponds to a tectonic sink connected with recent volcanic activity in the South-Campanian area (Phlegrean Fields, Ischia and Vesuvius). At its extreme north-west, the Isle of Ischia was early recognized as a volcano-tectonic horst by Rittmann¹. Previous data² concluded that its uplift ended around 300,000 yr, and that the Green Tuff formation, of which it is predominantly composed, had an age of some 700,000 yr. But tectonic and morphological studies, together with original tephrostratigraphical observations on the slope of the horst, led us to do some new K–Ar datings. These revealed that the 780 m uplift of the horst from the sea level is younger than 33,000 yr; the Green Tuff, which was considered to be the oldest volcanic unit in the island is now dated at 56,000 yr, its eruption following the dismantlement of a first volcanic complex now dated as not older than 130,000 yr.

Rittmann¹ recognized that the principal structure of the Isle of Ischia is a volcano-tectonic horst (Monte Epomeo, 787 m) and not a central volcano as had previously been believed^{3,4}.

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The horst of Monte Epomeo is part of a system of faults running north to south and from east to west, describing a quadrilateral between the villages of Lacco Ameno, Casamicciola, Barano and Panza (Fig. 1); it is formed of a trachytic ignimbrite: Green Tuff. Although of subaerial origin, this unit was totally covered by the sea before the uplift of the horst, as attested by the overlay of marine deposits and the existence of diffuse 'glaucinite' giving it its green colour. Rittmann considers Green Tuff as the oldest formation found outcropping on the island, on which all the products of subsequent volcanic activity occurring along the horst faults were deposited. K-Ar chronometric studies of volcanic units from the Isle of Ischia have dated Green Tuff at 6.0 ± 0.14 Myr (biotite)⁵ later corrected to 0.7 ± 0.1 Myr (sanidine and biotite)². According to these data, the most important phase in the activity of the island goes back to ~ 0.3 Myr and it would be around that period when the uplift of the horst ended. However, Evernden and Curtis⁶ have obtained a K-Ar dating of 83,000 yr on sanidine from a sample of Green Tuff, and an attempt at U-Th dating the Tuff of Citara^{7,8}, which is the product of an important explosive event of Ischia, gave a probable age of $41,500 \pm 3,000$ yr.

New tephrostratigraphical⁹, tectonic and morphological studies have been undertaken on the Isle of Ischia, as part of the research into the evaluation of volcanic risk in active volcanic regions. They revealed that lava units associated with breccia can be individually identified under the Green Tuff at the foot of the Western slope of Monte Epomeo (Rione Bocca), bearing witness to the existence of a phase of activity before the formation of the Green Tuff. However, they also reveal that the formation of the horst of Ischia is very recent, without doubt still active and directly correlatable with the historical volcanic activity attested by archaeological observations¹⁰ of which the latest manifestation is the Arso lava flow (December 1301–January 1302). The disparity of ages and the new data available prompted us to renew the chronometric study of the main eruptive phases on the island. The techniques used have been described elsewhere^{11,12}. Argon was analysed using a 180° , 6-cm radius, 500 V accelerating potential mass spectrometer operated in the static mode. The sensitivity of the machine was 4×10^9 atoms mV^{-1} using a $10^{11} \Omega$ resistor in the vibrating reed electrometer. For the ^{36}Ar peak, the output was typically 30–100 mV and the noise level was around 0.03 mV. The accurate correction for contamination necessary when dating such young rocks was obtained by avoiding any discrimination effect in the mass spectrometric measurements, that is, unspiking, and consequent successive measuring of 36 and 40 ion beam intensities for sample argon, atmospheric argon and the standard argon amount in the same working conditions in the mass spectrometer (with $<0.3\%$ fluctuation of any of the parameters—accelerating potential, arc voltage, electron beam current, background of the mass spectrometer with masses other than argon and the amount of argon present in the mass spectrometer when doing the comparison of sample argon with atmospheric argon)¹². In these conditions it is possible to date to within 10,000 yr (refs 12–14) lavas containing 1% of potassium. Therefore Ischia's rocks can be dated to within a few thousand years, being classified between latites and alkali-trachytes with a potassium content of at least 4%. This accuracy corresponds typically to a limit of detectability of 0.2% on the level of radiogenic ^{40}Ar . It makes possible a 10% accurate dating at a contamination level of 98%. Confirmation that such precision of measurement can be attained was provided by analyses of air argon samples measured at the same working point of the mass spectrometer; the uncertainty in $^{40}Ar/^{36}Ar$ ratio was 0.12% (2σ for a single analysis calculated from about 12 runs). Data on 11 samples from historic or radiocarbon dated volcanic units presented in Table 1 reveal a fairly good agreement and confirm the reliability of K-Ar data down to 0.2% of radiogenic argon. Among the analysed samples is one from the Arso flow from Ischia which erupted in AD 1302. Note that the dispersion observed for replicate measurements on the same sample was always within the range of the predicted error.

Sixteen samples of volcanic units of the Isle of Ischia were then dated. They were selected as a function of their stratigraphical position and corresponded to the different activities developed in the island: before the horst uplift, old volcanic remains outcropping on its periphery and volcanic units affected by the faults of the horst; recent volcanic activities developed along the faults of the horst, contemporaneously to the uplift. Ages obtained are reported in Table 2. All these results are in agreement with the available stratigraphical control: the trachyte of the Costa Sparaina (sample 2) flowed over the lava of the Selva del Napolitano (sample 3) both lying on Green Tuff (samples 8, 9) on the eastern flank of the horst; a trachytic block (sample 10) included in the Green Tuff and the lava flow at Rione Bocca (sample 11), overlain by Green Tuff, gave ages slightly older and coherent with other ages obtained for the oldest volcanic units outcropping on the Isle between 130,000 and 75,000 yr; also, samples 4, 5 and 6 corresponding to the products of the Campotese volcano and the Citara pumice level are directly superimposed. Moreover ages obtained on separated mineral phases (sanidine and groundmass) of the same unit, with various potassium contents and various levels of contaminant argon, gave the same ages within the limit of the predicted accuracy. Under the pumice deposit associated with the trachytic lava of Fondo d'Oglio crater (sample 1), which erupted during the historic period according to our K-Ar results ($<2,000$ yr), we have sampled shells in a palaeo-beach level to crosscheck our result with radiocarbon dating. We also found a piece of ceramic indicating that the pumice flowed over historical deposits, in good agreement with our result: this pottery could be attributed to the third century AD (G. Marinelli, personal communication).

Our K-Ar measurements confirm that Green Tuff, $56,000 \pm 4,000$ yr, is not the oldest volcanic formation outcropping on the Isle of Ischia. The dating of volcanic units between 130,000 yr (Castello d'Ischia lava, sample 16, $132,000 \pm 2,600$ yr; Monte di Vezzi lava flow, sample 15, $128,000 \pm 5,000$ yr) and 75,000 yr (dome of Monte Vico, sample 12) shows that volcanic centres were active before the Green Tuff's eruption. These centres correspond to volcanic units which are at present partly dismantled and visible at the

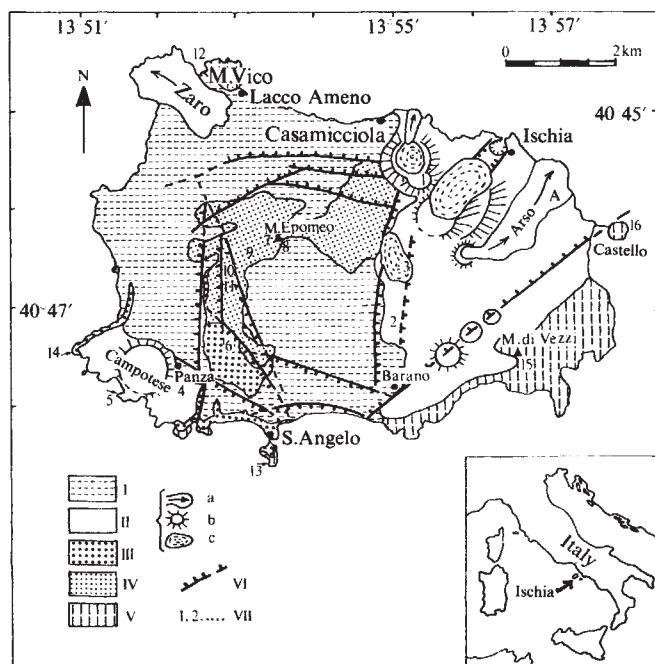


Fig. 1 Geological sketch map of the Isle of Ischia. I, scree and mud flows; II, volcanic units younger than 30,000 yr, a, lava flow; b, crater; c, dome; III, Citara Tuff; IV, Green Tuff of Monte Epomeo; V, formations of the first volcanic complex, dismantled; VI, faults; VII, samples location (same numbers as in Table 2 and the text).

periphery of the Epomeo's horst: this is also the case at Castello d'Ischia in the east, Monte Vico in the north, the peninsula of S. Angelo (sample 13, $97,000 \pm 5,000$ yr) in the south and Monte di Vezzi in the south-east. The first complex probably extended beyond the boundaries of the present island and dating indicates that it was built up over at least 60,000 yr. The major part of the old volcano has indeed been eroded and disappeared following former collapses and the Green Tuff's eruption. In the south-west part of Ischia, near Panza, the recent products of the complex volcanic centre of the Campotese are

superimposed on the remnants of old structures almost completely submerged and outcropping along the coast at Punta Imperatore (sample 14, $118,000 \pm 3,000$ yr), Capo Negro and S. Angelo. A level of pumice (the Citara Tuff) divides the old flows from the recent products of the Campotese⁹. Citara Tuff also overlaps the Green Tuff on the south-west slope of Monte Epomeo, where it is affected by the faults of the horst, and passes laterally into a facies of fine cinerites, which are intercalated with marine deposits. Hence, this pumice level (sample 6, $33,000 \pm 2,000$ yr), younger than the Green Tuff and the

Table 1 Analytical K-Ar data on volcanic rocks of known ages

	Material (K%)	⁴⁰ Ar* (%) (±0.2)	⁴⁰ Ar* (10 ¹⁰ atoms g ⁻¹)	K-Ar age (kyr)	Known age (yr)
A	Groundmass (6.00)	0.04	<3.2	<5.1	AD 1302
		0.00	<2.5	<4.0	
	Sanidine (9.00)	0.18	1.1	1.2±1.3	
		0.00	<1.3	<1.5	
B	Sanidine (10.55)	0.90	3.6	3.3±0.8	3,700±200 ¹⁴ C (ref. 15)
		1.18	4.7	4.2±0.8	
C	Sanidine (10.54)	10.43	17.0	15.4±0.7	Between 11,000 and 12,000 yr ¹⁴ C (ref. 15)
		8.80	15.5	14.1±0.7	
		3.55	16.7	15.1±0.9	
D	Sanidine (11.19)	8.90	40.7	34.8±0.9	Between 28,000 and 35,000 yr ¹⁴ C (ref. 16)
		9.70	39.8	34.0±0.9	
E	Groundmass (5.00)	0.05	<2.1	<4.0	AD 1944
	Leucite (15.00)	0.00	<7.9	<5.0	
F	Glass (5.00)	0.00	<1.1	<2.0	Late Roman decade, about 1,400 yr BP (ref. 17)
		0.08	<1.6	<3.0	
G	Groundmass (5.00)	0.25	0.8	1.5±1.2	Flank of modern Vulcano
		0.29	0.9	1.7±1.2	
	Sanidine (10.00)	0.00	<1.6	<1.5	
H	Groundmass (4.03)	0.20	2.1	5.0±5.0	Base of modern Stromboli
		0.25	2.7	6.5±5.0	
I	Groundmass (1.50)	0.00	<1.1	<7.0	AD 1971
	Mean value of 5 measurements ranging between -0.04 and 0.07% radiogenic level				
J	Groundmass (1.50)	0.00	<0.8	<5.0	Sixteenth century AD
	Mean value of 5 measurements ranging between -0.05 and 0.09% radiogenic level				
K	Groundmass (1.35)	0.60	1.9	13.0±4.3	7,800±300 ¹⁴ C (ref. 18)
		0.35	1.3	9.5±5.4	

A, Arso flow of Ischia (sample 36 I), drilled in the core of the flow at the international camping of Ischia Porto; B, trachytic dome of Caprara (sample CF 27), connected with Astroni crater, Phlegrean Fields, Campania, Italy; C, Pumice level (sample 36 O), connected with Neopolitan Yellow Tuff, Phlegrean Fields, Italy; D, Campanian Ignimbrite (sample 35 P) sampled at S. Agata dei Goti, Campania, Italy; E, AD 1944 Vesuvius tephritic flow (sample 35 H); F, Obsidian flow of Rocche Rosse (sample 28 X), Lipari Island, Italy; G, Grotta dei Palizzi trachytic flow (sample 38 T) southern flank of modern Vulcano, Aeolian Islands, Italy; H, Punta Frontone shoshonitic flow (sample 37 P) base of modern Stromboli (Filo dell'Arpa), Aeolian Islands, Italy; I, Mount Etna AD 1971 flow (sample 29 C) fine grained light grey facies of the core of the flow; J, Mount Etna (Sicily) sixteenth century AD flow (sample FLG 3) sampled in the quarry of Nicolosia; K, basaltic flow of Puy de la Vache (sample 30A), sampled at St Saturnin, Chaîne des Puys, Massif Central, France. The errors are calculated according to:

$$\frac{\sigma^2 \text{Age}}{\text{Age}^2} = \left(\frac{1-T}{T} \right)^2 \frac{\sigma^2 \text{Ar}}{\text{Ar}^2} + \frac{\sigma^2 \text{Cal}}{\text{Cal}^2} + \frac{\sigma^2 \text{K}}{\text{K}^2}$$

where T is the radiogenic argon proportion in total argon;

$$(\% \text{ error in radiogenic Ar})^2 + (\% \text{ error in calibration})^2 + (\% \text{ error in K})^2$$

Its value tends to $\pm 2\%$ when the radiogenic argon level becomes $>10\%$, taking into account a 0.5% (2σ) error on the calibration for a definite value of standards¹⁹ and an error of 1.5% on K content dosed by atomic absorption spectrophotometry. The error in the radiogenic argon was determined using the expression:

$$\left(\frac{1-T}{T} \right)^2 \left(\frac{\sigma^2 r}{r^2} + \frac{\sigma^2 r'}{r'^2} \right)$$

where r and r' are the $^{40}\text{Ar}/^{36}\text{Ar}$ ratios in sample argon and atmospheric argon. This expression is comparable with the formulation in refs 20–22. Because of the steadiness of the different argon signals measured in our machine and because of the simultaneous collection of ^{36}Ar and ^{40}Ar signals, the error in radiogenic ^{40}Ar corresponds to the errors in the measurements of isotopic ratios, and, essentially, to the error on ^{36}Ar measurements. In our case of very low radiogenic level, $r \sim r'$; the error on radiogenic argon can be written:

$$\left[2 \left(\frac{100 - ^{40}\text{Ar}^*}{^{40}\text{Ar}^*} \times \% \text{ error in } ^{40}\text{Ar}/^{36}\text{Ar} \right)^2 \right]^{1/2}$$

This is the major uncertainty in the calculated age because of the error magnification associated with the small proportion of radiogenic argon found in the analyses (0 – 10%).

Sample weight: 5 – 10 g per argon analysis; $^{40}\text{Ar}^*$ = radiogenic ^{40}Ar ; the following decay constants were used in the ages calculation²³: $\lambda_B = 4.962 \times 10^{-10} \text{ yr}^{-1}$, $\lambda_e = 0.581 \times 10^{-10} \text{ yr}^{-1}$, $^{40}\text{K}/\text{K} = 1.167 \times 10^{-4}$.

Table 2 Analytical K-Ar data on Ischia's lavas

Material (K%)	$^{40}\text{Ar}^*(\%)$	$^{40}\text{Ar}^*$ (10^{10} atom g^{-1})	Apparent age (kyr)
1 Groundmass (5.53)	0.20	0.5	1.0 ± 1.0
	0.27	0.8	1.6 ± 1.1
Feldspar (6.95)	0.15	0.8	1.0 ± 1.3
	0.29	1.5	2.0 ± 1.4
2 Groundmass (5.90)	0.39	2.3	3.7 ± 1.9
	0.65	2.9	4.5 ± 1.5
	0.31	1.9	3.0 ± 2.0
Feldspar (6.51)	0.42	2.9	4.1 ± 2.0
	0.47	3.9	5.6 ± 2.5
3 Groundmass (5.20)	1.28	5.7	10.2 ± 1.6
	1.17	4.9	9.0 ± 1.5
	1.26	4.1	7.6 ± 1.2
Feldspar (7.94)	1.42	8.6	10.3 ± 1.5
	1.45	8.7	10.4 ± 1.5
4 Sanidine (8.80)	2.86	21.7	23.7 ± 1.7
	2.82	21.5	23.5 ± 1.7
5 Sanidine (8.83)	1.66	25.9	28.1 ± 3.3
	1.80	27.3	29.6 ± 3.2
	1.95	27.0	29.3 ± 3.0
6 Sanidine (7.54)	3.55	25.6	32.5 ± 1.8
	3.86	26.4	33.5 ± 1.7
7 Sanidine (9.70)	3.28	57.4	56.7 ± 3.5
	3.51	55.0	54.2 ± 3.0
	1.79	58.8	58.0 ± 6.5
8 Sanidine (9.51)	2.69	56.5	56.8 ± 4.0
9 Sanidine (10.40)	2.59	55.7	51.5 ± 4.0
	3.03	61.6	56.7 ± 4.0
10 Groundmass (6.03)	0.60	71.1	113.0 ± 37.0
	0.54	62.3	97.0 ± 36.0
11 Groundmass (6.08)	3.34	85.7	135 ± 8.0
	3.42	82.4	130 ± 8.0
Feldspar (6.18)	8.19	78.7	122.0 ± 3.0
12 Groundmass (5.19)	16.39	41.4	76.0 ± 1.9
Feldspar (5.70)	15.55	43.5	73.0 ± 1.8
	16.51	45.2	76.0 ± 1.9
13 Groundmass (5.85)	2.09	60.6	99.0 ± 9.0
	2.20	61.3	100.0 ± 9.0
Feldspar (6.04)	6.17	59.1	95.0 ± 3.0
14 Groundmass (5.93)	7.95	72.7	117.0 ± 3.3
	13.92	73.1	118.0 ± 3.0
Feldspar (6.35)	18.41	77.0	116.0 ± 2.9
	21.52	81.2	123.0 ± 3.1
15 Whole rock (5.37)	4.96	71.6	128.0 ± 5.0
16 Groundmass (5.85)	23.02	80.9	132.0 ± 3.3

1, Fondo d'Oglio trachytic flow (sample 35 V); 2, Costa Sparaina trachytic flow (sample 35 Y); 3, trachytic lava at Selva del Napolitano (sample 35 W); 4, trachytic lava flow near Panza (sample 35 M); 5, trachytic lava, base of the cliff of Grotta del Mavone (sample 35 N); 6, Citara trachytic Tuff sampled at Ciglio (sample ISH 85); 7, Green Tuff at S. Nicola (sample 38 Eb), near the top of Monte Epomeo; 8, Green Tuff, real top of Monte Epomeo (sample 38 Ea); 9, Green Tuff at Pietra dell'Acqua (sample ISH 114 B); 10, trachytic block included in the Green Tuff, Rione Bocca (sample 36 D); 11, Rione Bocca trachytic flow (sample ISH 84), under the Green Tuff; 12, Monte Vico latitic dome (sample 35 J) at Baia S. Montano; 13, S. Angelo latitic dome (sample ISH 103), at the base of the mount; 14, trachytic lava, at the base of the cliff at Punta Imperatore (sample ISH 118); 15, Monte di Vezzi trachytic flow (sample 36 H); 16, Castello d'Ischia latitic lava (sample ISH 113). Same analytical conditions as presented in Table 1.

ancient lavas of the south and south-west part of the island, was deposited close to sea level before the formation of the horst of Monte Epomeo; consequently the amplitude of this uplift of at least 800 m can be dated back to <33,000 yr. The formation of the horst was accompanied by volcanic activity which developed at its periphery during the past 30,000 yr. In

particular, the north-east sector of Ischia, described by the eastern marginal fault of the horst of Epomeo and by the fault running south-west north-east Barano-Castello, appears to consist of pyroclastics and lavas, recently emitted by domes, explosion craters and spatter cones (for example, Selva del Napolitano lava, sample 3, $9,000 \pm 1,500$ yr; Costa Sparaina trachytic flow, sample 2, $4,000 \pm 1,800$ yr; Fondo d'Oglio trachytic flow, sample 1, $1,500 \pm 1,000$ yr). In this sector, it was possible, by dating, to link an important phase of activity to the prehistoric and historic period. Besides the formation of the flows and the trachytic domes, the pumice layers which extend over the whole eastern sector of the island also originated during this phase. The data from our study indicate that the volcanic and tectonic history of the Isle of Ischia is perceptibly different from that which was previously accepted and reveal the important seismic and volcanic risks in this zone. The age of the volcanic activity of Ischia is much younger than was thought. From ~130,000 yr ago, a first complex was built which was partly eroded and dismantled by a tectonic phase which finished by a collapse, following the eruption of the Green Tuff, dated at $55,000 \pm 3,500$ yr. The uplift of the volcano-tectonic horst of Monte Epomeo is <33,000 yr old, as given by the age of the Citara Tuff. It occurred at the same time that the modern volcanic activity developed.

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Unusual distribution of biological markers in an Australian crude oil

R. P. Philp & T. D. Gilbert

CSIRO Division of Fossil Fuels, PO Box 136, North Ryde, New South Wales, 2113, Australia

The stereochemical configuration of hopane-type triterpanes in source rock extracts or crude oils is normally determined by the maturity of the sample¹. Naturally occurring precursors of the hopane hydrocarbons are dominated by the $17\beta\text{H}$, $21\beta\text{H}$ stereochemistry with only one diastereomer at the C22 position in the C_{31} and higher homologues². Note that the $17\beta\text{H}$, $21\beta\text{H}$ - C_{28} homologue has never been reported in any sample. Mature crude oils in general contain hopane hydrocarbons dominated by the thermally more stable configuration of $17\alpha\text{H}$, $21\beta\text{H}$ stereochemistry and a mixture of the two diastereomers at the

C22 position for C₃₁ and higher homologues in the ratio of ~3/2. We present here evidence for the presence of an unusual distribution of hopanes in an Australian crude oil. A study by computerized gas chromatography-mass spectrometry (C-GC-MS) designed to determine whether different oils from the same basin have similar sources has revealed the presence of the thermally unstable 17 β H, 21 β H isomers of hopane-type triterpanes in conjunction with C22 diastereoisomers in the unusual ratio of 1:9. We propose that there are two possible explanations for this unusual distribution of the triterpanes. (1) The oil is immature or (2) these compounds have accumulated in the oil as it migrated through coals in the region of the reservoir which are also known to contain the unusual hopane distribution.

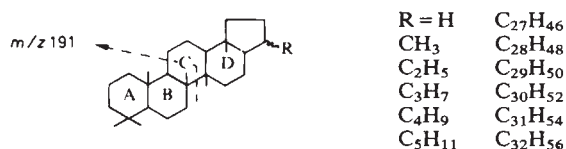


Figure 1a, which is a portion of the m/z 191 chromatogram obtained from the GC-MS analysis of an Australian crude oil, illustrates the use of the technique of single-ion monitoring (SIM) to determine the distribution of the triterpanes³. This ion corresponds to the A/B ring fragment of the hopane structure shown above. Examination of complete spectra showed that the predominant hopane components in this sample (1, 2, 5-10) all have the 17 α H, 21 β H stereochemistry as has been previously reported for crude oils¹. The ratio of the C₃₁ 22S and R isomers is ~60/40, previously reported by Seifert *et al.*⁴ to be the equilibrium ratio for these isomers in crude oils. The second eluting isomer possesses the naturally occurring stereochemistry (22R). It was previously thought that crude oils with a 22R/22S ratio of >1 have never been observed since such a distribution would indicate lack of sufficient maturity for petroleum generation⁴.

We now report the first observation for Australian crude oils of a higher proportion of the C₃₁-22R diastereomer than the C₃₁-22S diastereomer. This unusual distribution has been observed in at least two oils from the Gippsland Basin, one from the Surat Basin and one from the Carnarvon Basin but is most pronounced in the Turrum 2 oil discussed in detail here. Thus from a total of ~40 oils examined, 10% had a higher concentration of C₃₁-22R epimer than the C₃₁-22S epimer and several others had a 1:1 ratio of the 22R and S isomers. Shi Ji-Yang *et al.*⁵ have recently reported a similar occurrence in one oil from China and Seifert *et al.*⁴ reported oils from one basin where the 17 α (H), 21 β H 22R:22S ratio was greater than the proposed equilibrium ratios and where ratios as low as 1.2 were observed in the least mature oils. However, the present study suggests that such a distribution is not uncommon in crudes from several Australian basins.

The most striking example of this unusual ratio was observed in an oil from the Turrum well in the Gippsland Basin for which the m/z 191 chromatogram is shown in Fig. 1b. In this example the ratio for 22S and R is 10/90 compared with the 60/40 predicted by Seifert *et al.*⁴ for mature oils, where the 22S and R isomers are supposedly in thermodynamic equilibrium. Furthermore, the ratio for the C₃₂-22S and R diastereomers is analogous to the C₃₁ ratio in that the 22R diastereomer is present in higher concentrations than the 22S diastereomer. Examination of complete spectra taken over the peak from the second eluting isomer showed no evidence for the presence of gammacerane which has been reported to co-elute with the C₃₁-22R-homohopane on non-polar columns⁵. Comparison of this oil with that taken from a reservoir 2,000 ft deeper in the same well illustrates a significant difference in distribution of the hopanes (Fig. 1c). The deeper oil has a more characteristic hopane distribution pattern dominated by the 17 α H, 21 β H-C₂₉ component and with the C₃₁-22S and R isomers present in a

53/47 ratio. It is generally thought, though not unequivocally proven, that the oils from this part of the basin have the same or very similar sources⁶. If this is the case for these two oils, what explanations are available for the unusual ratio of C₃₁-22S and R diastereomers in the shallower oil plus several other differences in individual hopane distributions?

The location of the Gippsland Basin and the onshore Victorian brown coal deposits prompted a re-examination of the triterpanes present in Yallourn lignite. The sample of Yallourn lignite used in this study was taken from the onshore area of the Gippsland Basin. Most of the offshore areas of the basins contain relatively high proportions of dispersed coaly material

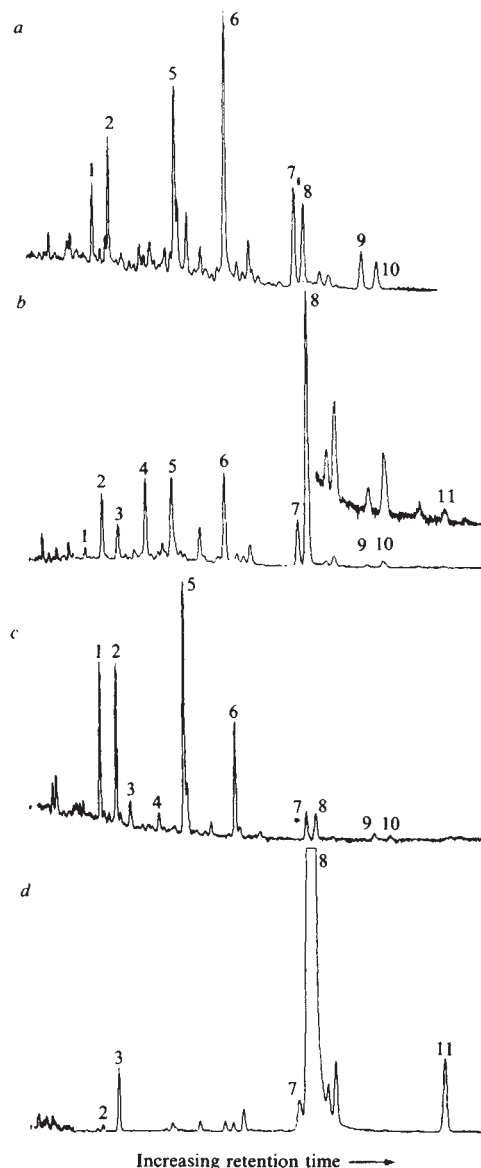


Fig. 1 Partial single-ion chromatograms of m/z 191 for Kingfish (a) and Turrum (b, 5,177 ft; c, 7,624 ft) oils from the Gippsland Basin and an extract from Yallourn lignite (d). Samples were analysed on a Finnigan 4023 GC-MS system. GC conditions were: 40 m fused silica WCOT column programmed from 10 to 200 °C at 20 °C min⁻¹ and then 4 °C min⁻¹ to 280 °C. The mass spectrometer was operated in multiple-ion detection mode at an electron energy of 70 eV and ion current of 250 μ A. Peak identification: 1, 18 α (H)-22,29,30-trisnorhopane; 2, 17 α (H)-22,29,30-trisnorhopane; 3, 17 β (H)-22,29,30-trisnorhopane; 4, 17 α (H), 21 β (H)-28,30-bisnorhopane; 5, 17 α (H), 21 β (H)-30-norhopane; 6, 17 α (H), 21 β (H)-hopane; 7 and 8, 22S and R 17 α (H), 21 β (H)-30-homohopane; 9 and 10, 22S and R 17 α (H), 21 β (H)-30,31-bishomohopane; 11, 17 β (H), 21 β (H)-30-homohopane. Inset in b is $\times 10$ magnification.

that is very similar to the onshore lignite and brown coal deposits although of a slightly higher maturity. Figure 2a shows a cross-section through this part of the Gippsland Basin and illustrates the presence of non-marine sands, shales and coals in the reservoir areas of the Turrum well. Figure 2b shows the stratigraphical sequence through the Turrum 2 well, again indicating the presence of interspersed coals in the reservoir zones. In this study the Yallourn lignite is used as a concentrated example of the type of material that is typically dispersed throughout the basin.

Van Dorsselaer *et al.*⁷ found that the major triterpane component in Yallourn lignite is the $17\alpha(\text{H})$, $21\beta(\text{H})$ - $22\text{R}-\text{C}_{31}\text{H}_{54}$ homohopane diastereomer. The m/z 191 chromatogram (Fig. 1d) for the hydrocarbon fraction from a sample of Yallourn lignite demonstrates that in addition to the $17\alpha(\text{H})$, $21\beta(\text{H})$ - $22\text{R}-\text{C}_{31}$ -homohopane, $17\beta(\text{H})$ - C_{27} -trisnorhopane and $17\beta(\text{H})$, $21\beta(\text{H})$ - C_{31} -homohopane were also present as very minor components. Note that this sample of lignite is only being used to demonstrate the unusual hopane distribution; it is unlikely that it is specifically responsible for the unusual hopane distributions in the shallow Turrum oil because it does not contain the C_{32} -homohopanes. Detailed analysis of the Turrum 2 oil showed that the two $17\beta(\text{H})$ -hopanes mentioned above were also present in the oil. The presence of $17\beta(\text{H})$ -hopanes in crude oils is in itself a very unusual observation. It is generally considered that at the maturity levels required for the formation of petroleum, the $17\beta(\text{H})$ isomers will be converted into their $17\alpha(\text{H})$ analogues, although trace amounts of the $17\beta\text{H}$ C_{27} -hopane are present in genuine source rocks and petroleum⁸. If this is the case, the only explanation for the presence of the two $17\beta(\text{H})$ hopanes plus the predominance of the $22\text{R}-\text{C}_{31}$ -homohopane seems to be that they are derived directly from an immature brown coal source, similar to the Yallourn lignite, which has been in contact with the oil.

There are alternative explanations for this unusual distribution of triterpanes. MacKenzie *et al.*^{9,10} have proposed that an oil can appear immature on the basis of one type of biological marker parameter but be apparently mature, or 'normal', in terms of another type of measurement. In other words, a measurement of the extent to which one reaction type has occurred will correspond to different values of a measurement based on a different reaction type. Determination of the % $20\text{S}/20\text{R} + 20\text{S}-24\text{-ethyl}-5\alpha(\text{H}), 14\alpha(\text{H})17\alpha(\text{H})$ -cholestane for the two Turrum oils from the m/z 217 chromatogram gives values of 23% and 43% for the shallow and deep oils, respectively. The value for the shallow oil is similar to that observed by Shi Ji-Yang *et al.*⁵ for samples of immature Chinese oils.

The immaturity of the Turrum 2 oil based on the % $20\text{S}-\text{C}_{29}$ -sterane combined with the hypothesis of Mackenzie *et al.*^{9,10} would suggest that the Turrum 2 oil is an immature oil similar to those first observed by them in samples from China. Whilst agreeing in general with their hypothesis, we feel that the Turrum 2 oil, and possibly other Australian oils are exceptions to this hypothesis. We propose that the unusual distribution of the 22R and $\text{S}-\text{C}_{31}$ -homohopanes in the Turrum 2 oil arises as a result of oil coming into contact with relatively immature brown coal zones in the reservoir areas. Many of the reservoirs in the Gippsland Basin contain significant quantities of dispersed coaly material (Fig. 2a, b) and hence the oil would be in contact with this material for a relatively long period of time. The strongest pieces of supportive evidence for this theory are the presence of the $17\beta(\text{H}), 21\alpha(\text{H})-\text{C}_{27}$ and C_{31} -hopanes in both the oil and a sample of onshore Yallourn lignite and the predominance of the $22\text{R}-\text{C}_{31}$ -homohopane in both the oil and the extract of the Yallourn lignite. The results of this study suggest that, in certain cases, the biological markers in crude oils may not always be derived directly from major source rocks but may be acquired during migration or after accumulation in the reservoir. Such an observation may be unique for Australian basins where the content of coal is generally much higher than for other oil-bearing basins around the world.

We thank Esso Australia Ltd for samples of the Gippsland

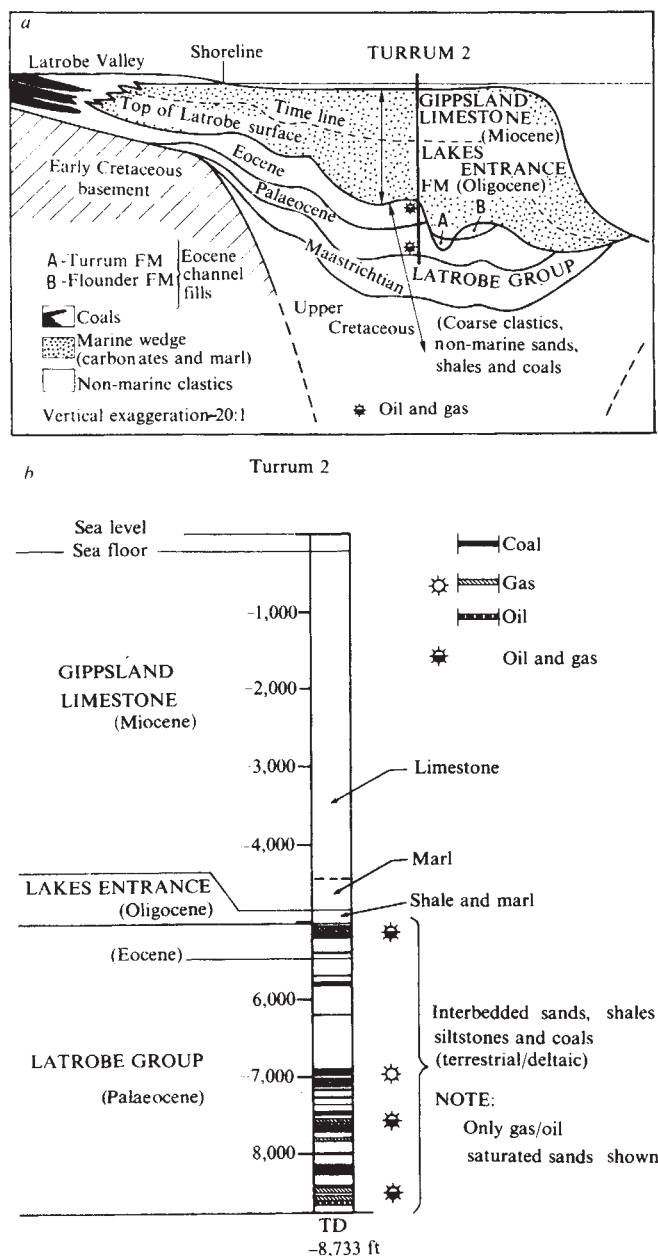


Fig. 2 a, Cross-section through part of the Gippsland Basin in the vicinity of the Turrum 2 well. b, Stratigraphical sequence through the Turrum 2 well.

oils and Dr J. R. Maxwell for his constructive criticisms and for providing preprints, also Esso Australia Ltd and Hematite Petroleum Pty Ltd for permission to reproduce the diagrams shown in Fig. 2. Fig. 2a was originally drawn by A. D. Partridge of Esso Australia Ltd.

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The representation of auditory space in the mammalian superior colliculus

A. R. Palmer & A. J. King

National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

The superior colliculus (SC) is involved in orienting responses to sensory stimuli¹ and contains detailed sensory and motor maps¹⁻³. The visual and somatosensory maps have been well documented⁴⁻¹⁵ and are topographic representations of the primary receptor surfaces. However, the formation of an ordered map of auditory space is rather more problematic as it requires either reorganization of monaural information or combination of information from both ears^{16,17}. Nevertheless, such an ordered map has been demonstrated in the midbrain of the barn owl¹⁸ and there have been indications that a similar map occurs in mammals^{7-9,12,13,19-21}, although a recent study suggests that this is not the case in the cat²². Here we report briefly the results of our study, showing that auditory space is precisely represented in the deep layers of the guinea pig SC. This map of auditory space resembles the visual map in that cells responding to sounds in the anterior contralateral field are located rostrally, while those responding to sounds in the posterior contralateral field are located in caudal SC.

We have recorded auditory evoked responses of 155 cells throughout the deep layers of the guinea pig SC. After anaesthetizing the animals²³, the tracheae were cannulated and rectal temperature was maintained at 37 °C. A craniotomy was performed above the cortex overlying the SC and recordings were made with cortex intact using glass-coated tungsten microelectrodes. The animals were held from behind in a normal position with a minimal head-holder screwed to the skull. The pinnae, which were reflected by the initial incision, were carefully repositioned by sutures. The animal was placed at the centre of an anechoic chamber on a small table around which, level with the interaural plane at a radius of 1.1 m, was an array of loudspeakers at 22.5° intervals. A small flashing light was used to locate the centre of the visual receptive fields (RFs) of cells in the superficial layers. Electrodes were positioned to traverse the representation of the horizontal visual meridian so that auditory fields, if in register, would occur within the arc of speakers. A 100-ms burst of noise with a bandwidth of 100–20,000 Hz was presented repeatedly once every 1–2 s, while

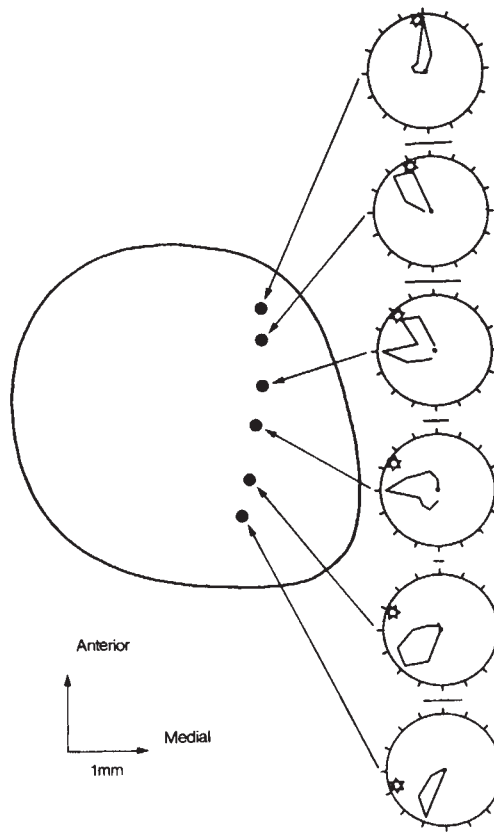


Fig. 2 Surface view of the guinea pig SC showing the location of six electrode penetrations at successive positions along the rostro-caudal axis. Polar diagrams are shown for six SC cells recorded in the electrode penetrations indicated. Each graph shows only responses at one sound level, at which the field was well defined, near threshold for each cell. Bars indicate 0.5 spikes per stimulus. Stars indicate the position of the RF of superficial layer visual cells. The cell showing two lobes could not be allocated a single preferred sound location and does not appear on the subsequent figure.

the recording electrode was being advanced through the deep layers of the colliculus. Once a single cell was isolated, its average discharge was measured in response to sound from the 11 speakers. Electrolytic lesions were placed in each electrode track to enable accurate identification of the recording site from histological reconstructions.

Of the cells we have studied, 75% showed a preference for sound originating from a particular direction. The effect of stimulus intensity on this directional preference was examined

Fig. 1 Number of spikes evoked from a SC cell per presentation of a 100-ms wideband noise stimulus, as a function of the horizontal location of the sound source and its level. Each value plotted on the radial axis of the polar graph is the mean response to 32 presentations. The repetition rate of the stimulus was decreased until an optimal response was obtained which usually occurred at one presentation every 1–2 s. In *a* the repetition rate was one every 1,800 ms and in *b* one every 1,600 ms. Only spikes occurring during a time window 10–70 ms after the stimulus onset were counted to minimize the contribution of spontaneous discharges. The bar represents a radial extent equal to 0.5 counts per presentation. It is clear that the discharge rates measured are extremely low. Although habituation in the SC deep layers has been well documented, increase in the inter-stimulus interval beyond those used did not increase the discharge probability. Such poor responsiveness has been attributed by several authors to the use of inappropriate stimuli. For example, cells in deep SC are known to be more responsive to moving stimuli which were not possible with the present apparatus. The angle of the polar plot represents the angular horizontal position of the loudspeaker. The star symbol indicates the position of the centre of the visual RF of cells in the superficial layers encountered in the same electrode penetration. Note that the highest sound level did not evoke the biggest response. Such non-monotonic responses were frequently encountered.

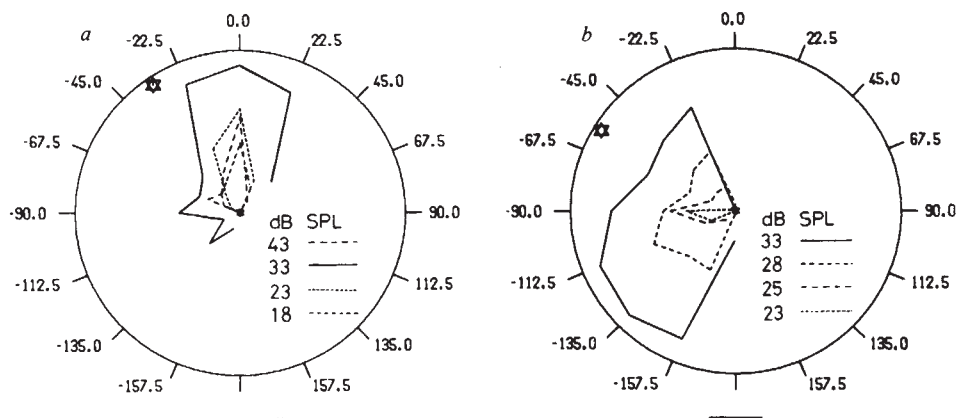
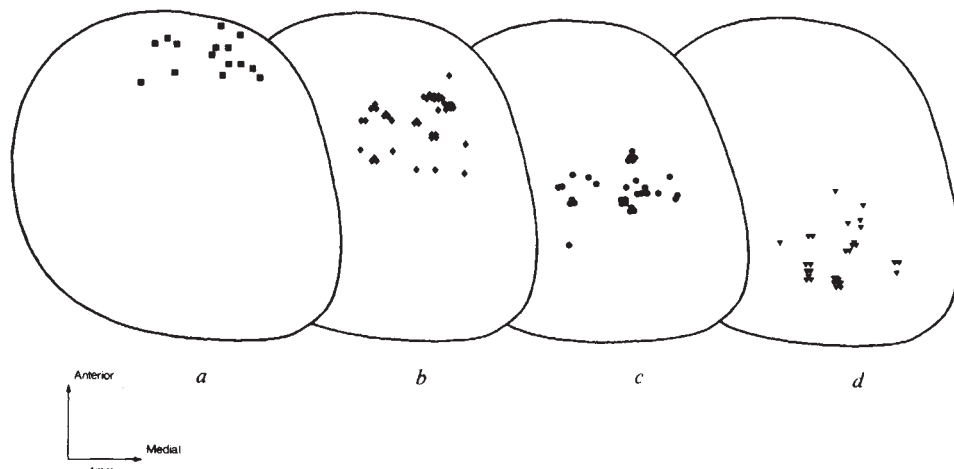


Fig. 3 Four identical surface views of the guinea pig SC. *a* Shows the position of electrode penetrations in which visual responses in the superficial layers were observed, but no auditory responses could be detected in the deeper layers. *b-d* Show respectively the position of cells which showed a preference for sounds located at 0–45°, 67.5–90° and 112.5–157.5°.



in 92 cells. Of these, 39% were narrowly tuned to a particular direction regardless of the sound level used, whereas the directional tuning of the remaining 61% broadened markedly with increasing stimulus intensity. Two examples of cells showing these characteristics are illustrated in Fig. 1. The cell shown in Fig. 1*a* responded to sound emanating from a limited spatial angle in front of the animal, even when noise levels were more than 25 dB above its minimum threshold. Figure 1*b* shows the response of a cell which preferentially responded to sounds from a particular direction only for noise levels near threshold. For levels even a few dB above this, however, the range of effective sound locations expanded, particularly in the caudal direction, in some cases occupying the whole contralateral field. The only cells which were driven by sounds in the ipsilateral auditory field were those with preferred sound locations at or near 0° (see Fig. 1*a*). Of 54 penetrations in which auditory responses were observed, more than one cell was found in 40. Tuned cells in a single penetration responded optimally to sounds from the same direction.

As the site of the recording electrode was moved from the anterior to the posterior part of the superior colliculus, the location of sounds to which the cells responded most vigorously shifted from the anterior to the posterior auditory field of the animal. This spatial representation of auditory space is analogous to the visual map over the collicular surface. Figure 2 shows data from a single animal in which responses from six electrode positions were analysed. The cells at each electrode site attended to a narrow region of space and formed an ordered array including the entire hemifield. Whether the vertical axis is also represented in the medio-lateral axis of the colliculus remains to be investigated.

That cells in a particular region of the superior colliculus are tuned to a specific location can also be illustrated using pooled data from different animals. Figure 3 illustrates the collicular position of 117 cells which showed a preference for sound location, grouped according to spatial location of their auditory receptive fields. These data indicate a reasonably strict topographical mapping of the auditory projection onto the deep layers of the SC over most of its rostral-caudal dimension (pooling of data and other unavoidable inaccuracies obscures the monotonic progression shown in Fig. 2). We did not find any cells responsive to auditory stimulation in 15 penetrations through the rostral pole of the SC (as indicated in Fig. 3*a*) even though these tracks certainly traversed both deep and superficial layers (in which visual responses were recorded).

Having demonstrated a well organized auditory map in the SC an obvious question is whether this map is in register with those of other modalities. Certainly, the visual maps found in other rodents^{4,8–10,12–14} resemble the topographic arrangement shown in Fig. 3. More direct evidence is, however, provided by our own visual RF data. In each penetration we routinely determined the centre of the RFs of cells in the superficial layers. The positions of these were in register with the auditory

fields ($\pm 11^\circ$) in 43% of cases, but often severe discrepancies of up to 60° occurred, particularly for auditory fields in front or behind the animal. Three main factors contributed to this discrepancy: (1) the abnormal position of the eye due to anaesthesia (on average the eye was 10.5° forward and 22° downward of the central orbit position). (2) The rostral and caudal curvature of the SC caused sampling from different topographical regions in the superficial and deeper layers. (3) The absence of auditory responses in the rostral SC. Allowing for these it appears that when the eye is in a normal position the maps of visual and auditory space are in register. Registration has also been indicated by previous authors^{7–9,12,13,19–21}, but final resolution of this question awaits detailed simultaneous mapping of the different modalities.

It is generally accepted that the auditory system localizes sound by comparing time and intensity differences between the two ears, although significant monaural localization has been demonstrated¹⁷. As we have shown here, cells within the SC are sensitive to the cues associated with a specific spatial location and are arranged to produce an internal representation of these locations. We do not yet know either the cues used by the guinea pig in localizing sound or the developmental strategies used in ordering the neural connections. For these reasons directional hearing is and has been a particularly challenging research problem, the ultimate solution of which will depend on understanding the nature of the excitatory and inhibitory interactions between the two ears and the detailed circuitry of the auditory projections.

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Metamorphosis of the insect nervous system: changes in morphology and synaptic interactions of identified neurones

Richard B. Levine* & James W. Truman

Department of Zoology, University of Washington, Seattle, Washington 98195, USA

The nervous system of holometabolous insects directs the behaviour of three radically different stages, the larva, pupa and adult. In some cases, larval neurones degenerate during metamorphosis and are replaced in the adult by neurones derived from retained embryonic neuroblasts^{1,2}, but many larval neurones are retained and undergo a morphological and synaptic reorganization which allows them to perform new functions in the adult^{3,4}. This cellular rearrangement, however, creates a problem in that the developing adults continue to display pupal-like behaviour even after the neurones attain their adult form. We show here that in the tobacco hornworm, *Manduca sexta*, the adult function of a neurone is prevented from being expressed precociously by the persistence of inhibitory influences that are removed abruptly at adult emergence.

We have investigated the changes that occur during metamorphosis in the relatively simple abdominal portion of *M. sexta*. During adult development several external muscle groups differentiate and are innervated by motoneurones whose larval targets have degenerated shortly after the larval-to-pupal molt⁴. The motoneurone MN-1 controls dorsal external oblique muscle DEO 2 in the larva, but loses this target in the pupal stage, innervating a new dorsal external muscle (DE 4) in the adult (Fig. 1). During this transition the cell undergoes considerable anatomical reorganization³. Figure 1 shows that the growth of new processes was most vigorous between days 4 and 12 of development and the adult morphology was essentially established by day 14.

The structural reorganization of MN-1 is correlated with changes in the pattern of synaptic inputs to the neurone. We chose to study a stretch receptor, specifically SR-3, which is located bilaterally in each abdominal segment and is present throughout the life of the insect⁵ (Fig. 1). Each sensory cell sends an axon into the central nervous system (CNS) and responds to stretch of its segment with a burst of action potentials⁶. Its central processes, which are similar at all stages, branch in the dorsal region of the neuropil and are confined totally to the hemiganglion in which they enter (Fig. 1).

During the larval stage we observed that MN-1 received excitatory input from the SR-3 ipsilaterally to its axon and target muscle (Fig. 2). A single electrical stimulus to the stretch receptor resulted in a constant-latency excitatory postsynaptic potential (e.p.s.p.) in the motoneurone. After subtracting the portion of this latency due to conduction of the SR-3 spike into the CNS (obtained by recording from SR-3 in the periphery and within the CNS), the remaining latency was less than 1 ms, a delay consistent with the pathway being monosynaptic. The processes of both neurones extended through similar regions of the dorsal neuropil and simultaneous cobalt backfills revealed areas of potential contact. This pathway remained unchanged throughout metamorphosis.

The larval MN-1 was inhibited by the SR-3 contralateral to its target. Stretch of this SR-3 or trains of electrical stimuli to its axon caused inhibition of tonic MN-1 activity, which was associated with a hyperpolarization and a conductance increase in the motoneurone (Figs 2, 3). The inhibitory postsynaptic

potential (i.p.s.p.) evoked by a single spike in the contralateral SR-3 had a long, variable latency and decreased rapidly in an all-or-none fashion during repeated stimulation. The terminal branches of the contralateral SR-3 were anatomically separated from the main dendritic arborization of MN-1. The only point of possible contact between the two neurones—the neurite leading from the soma of MN-1—passed ventral to the processes of SR-3. These data suggest that the inhibitory influence of the contralateral SR-3 is mediated by a polysynaptic pathway.

In the early pupal stage (days 1 and 2 after pupal ecdysis), the contralateral SR-3 continued to inhibit MN-1, but the relationship changed dramatically during metamorphosis. In the adult moth (1–2 days post-emergence), trains of stimuli to the contralateral SR-3 evoked a large depolarization and firing of the motoneurone (Fig. 3). Single stimuli resulted in an e.p.s.p. with a short, constant latency (Fig. 2). The i.p.s.p. which resulted from stimulation of the same SR-3 in earlier stages was not revealed by hyperpolarization or depolarization of the adult MN-1. On reexamination of the pathway in the larva, it was found that hyperpolarization of MN-1 caused reversal of the i.p.s.p. in a single step, and failed to reveal a hidden, short latency excitatory component in the interaction (Fig. 2). This change in the functional relationship of SR-3 with MN-1 coincides with the morphological changes shown by the motoneurone. The proximity of the new adult-specific dendrites to the terminal branches of the contralateral SR-3, together with the short, constant latency of the new e.p.s.p., suggests that the SR-3 makes direct contacts with these new dendrites.

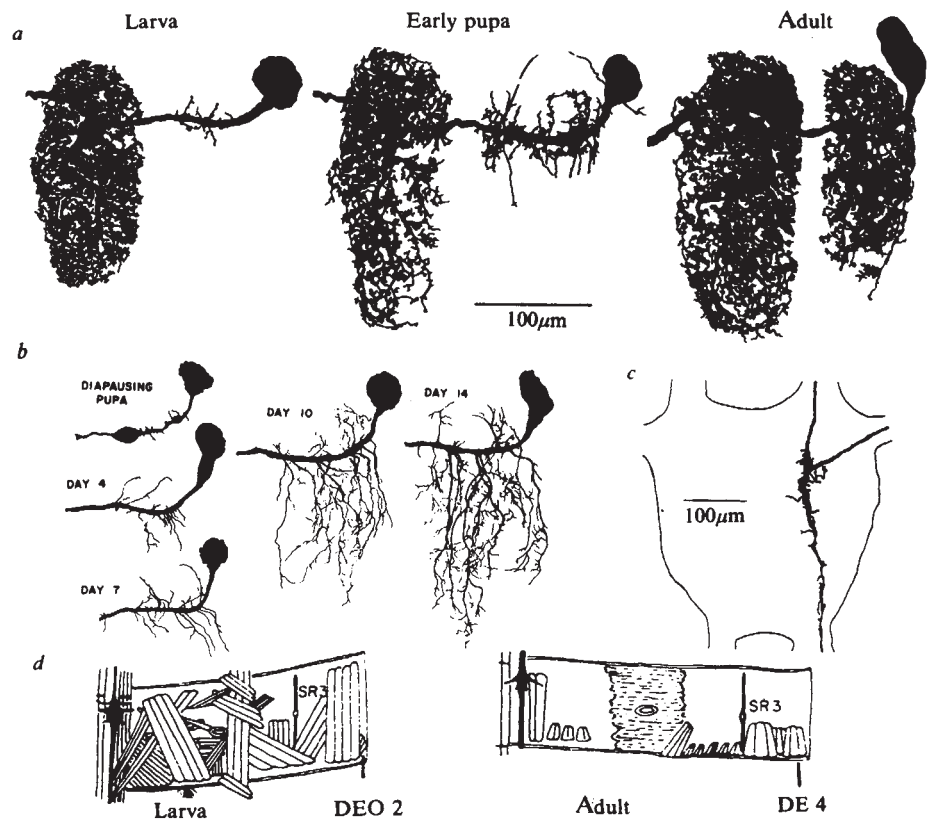
MN-1 attains its adult morphology and has full control of its new target 3–4 days before adult emergence. Despite these changes, however, the behaviour of the animal (which is covered by a pupal cuticle), is pupal in character until the time of adult emergence, when its behaviour abruptly switches to that of the adult⁷. Certain behaviours such as the defensive gin-trap reflex are unique to the pupal stage⁸, and new neuronal pathways must develop without disrupting them. On the last day of adult development, individual SR-3 impulses evoked a biphasic response in the contralateral MN-1, a short latency e.p.s.p. followed by an i.p.s.p. (Fig. 2). Occasionally the e.p.s.p. was sufficient to evoke an action potential in MN-1, but trains of stimuli or stretch of the receptor resulted in inhibition of the motoneurone (Fig. 3). The inhibitory component disappeared abruptly following adult emergence. Within 30 min of emergence, bursts of stimuli to the contralateral SR-3 evoked a pure excitatory response in MN-1 and single stimuli produced a short latency e.p.s.p., without an i.p.s.p. (Fig. 2). Furthermore, depolarization of the motoneurone revealed no i.p.s.p., and spontaneous activity was not inhibited during the period formerly associated with the i.p.s.p.

The functional and anatomical reorganization of MN-1 is reasonable in terms of its behavioural roles in the three stages of insect life. Both the larval and adult targets of MN-1 are near the dorsal midline of the abdominal segment. In the larva and pupa many behaviours depend on the ability of the abdomen to flex laterally. The right and left targets of MN-1 would be antagonists in this situation. Within a day after adult emergence, however, the abdomen of the moth becomes relatively rigid, and flexion is confined to the dorso-ventral plane. This lack of lateral movements makes asymmetric SR-3 inputs relatively pointless, as the only movements that are possible are ones for which the two DE 4 muscles must contract or relax together. Indeed, in the adult the right and left MN-1s often fire together in synchronous bursts. This synchrony is not due to direct coupling between the cells, which suggests that they share numerous common inputs.

A simple model for the change in the relationship of MN-1 to the contralateral SR-3 is shown in Fig. 3. In the larva, the contralateral SR-3 inhibits MN-1 through an unidentified interneurone. During adult development MN-1 grows new dendritic branches and accepts excitatory synapses from the contralateral SR-3. Before adult emergence, however, this excitatory connection is not behaviourally effective because the persisting parallel

*Present address: Department of Biology, Rice University, Houston, Texas 77001, USA.

Fig. 1 Structural reorganization of MN-1. *a*, Camera lucida drawings of MN-1 in the larval, early pupal and adult stages. Neurons were stained by passing positive current through an intracellular micropipette filled with 10% cobalt nitrate, and the stain enhanced in whole mount with a modification of the Timm's silver intensification procedure¹¹. In the larval and adult stages, MN-1 was identified by correlating activity recorded intracellularly from the motoneuron with that recorded extracellularly or intracellularly from the target muscle. In addition, action potentials in the MN-1 axon may be identified unambiguously in extracellular records from the third branch of the 'dorsal' segmental nerve, which allows MN-1 identification in the pupal stage. Note that during adult development MN-1 acquires a new dendritic field ipsilateral to its soma. *b*, Growth of the dendritic field ipsilateral to the MN-1 cell body. Neurons were stained at various times during adult development by back-diffusion of cobalt through the cut axon, followed by silver intensification of sectioned material. Although the period of adult development is 18 days, dendritic growth is essentially complete by day 14. *c*, Morphology of the stretch receptor (SR-3) sensory cell within the abdominal ganglion of an adult. The cell's morphology is similar throughout the animal's life. Note that its processes do not cross the midline of the ganglion. Although not shown, the processes of the sensory cell extend into the adjacent abdominal ganglia. *d*, Abdominal musculature in the larva and adult. In each case half of one abdominal segment is shown as though the animal had been cut along the dorsal midline and pinned open. The ventral nerve cord is on the left. The intersegmental muscles have been dissected away to reveal the external muscles, all of which die at the end of the larval stage and are replaced by newly generated muscles in the adult. The motoneuron MN-1 innervates a dorsal external oblique muscle (DEO 2) in the larva, and a dorsal external muscle (DE 4) in the adult. The right-hand stretch receptor (SR-3) is shown at each stage.

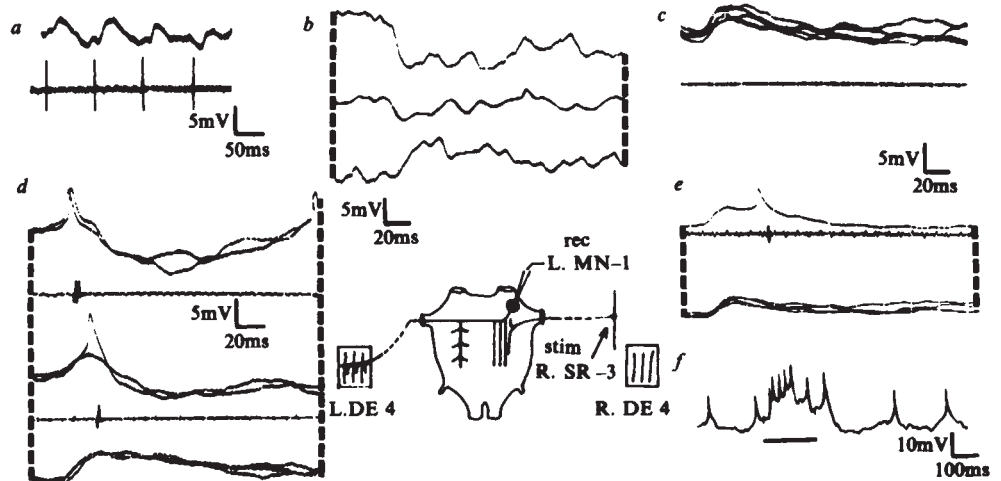


inhibitory pathway is always co-activated. An alternative interpretation is that the inhibitory synapse of the larval stage becomes excitatory and passes through a bifunctional stage⁹. Our data suggest, however, that the larval pathway is polysynaptic, while the adult pathway is direct.

The inactivation of the inhibitory pathway may be triggered

by eclosion hormone, which is released at the end of adult development and acts on the nervous system to release the emergence behaviours¹⁰. A second possible signal is the steroid 20-hydroxyecdysone, the reduction of which late in adult development triggers the death of many motoneurons and interneurons (J.W.T. and L. M. Schwartz, in preparation).

Fig. 2 Influence of SR-3 on MN-1 at different stages in the life cycle. *a*, The larval MN-1 is excited by the SR-3 ipsilateral to its target muscle. Each action potential in the SR-3 sensory cell (bottom, recorded near the sensory organ) causes an e.p.s.p. in the ipsilateral MN-1 (top). *b*, Inhibition of the larval MN-1 by the contralateral SR-3. A single electrical stimulus to the contralateral SR-3 (at the start of each trace) caused an i.p.s.p. in MN-1. The i.p.s.p. was enhanced by depolarization of MN-1 (top), and reversed by hyperpolarization (bottom). Note that a short-latency e.p.s.p. is not present (compare with *c-e*). *c*, Influence of the contralateral SR-3 on MN-1 in an adult 30 h after emergence. Intracellular (top), and extracellular (bottom) records from MN-1. Single stimuli to the SR-3 (at the start of the trace) evoked a constant-latency e.p.s.p. in the contralateral MN-1 (multiple trials, compare with *b*). *d*, Traces same as in *c*. About 5 h before adult emergence, single stimuli to SR-3 evoked a biphasic response in the contralateral MN-1. The later, inhibitory component was enhanced by depolarization of the motoneuron (top), and reversed by hyperpolarization (bottom). The early component may lead to an action potential (also visible in the extracellular records). *e*, Single stimuli to SR-3 30 min after emergence evoked only the short-latency depolarizing response in the contralateral MN-1 (bottom). Depolarization of the motoneuron revealed no inhibitory component (top). Note that action potentials were not blocked during the period formerly associated with the i.p.s.p. *f*, Bursts of stimuli to the contralateral SR-3 (bar) 30 min after emergence excited the MN-1. The insert shows the experimental set-up for *b-e*. The adult morphology of MN-1 is drawn schematically. rec, Recording electrode; stim, stimulating electrode.



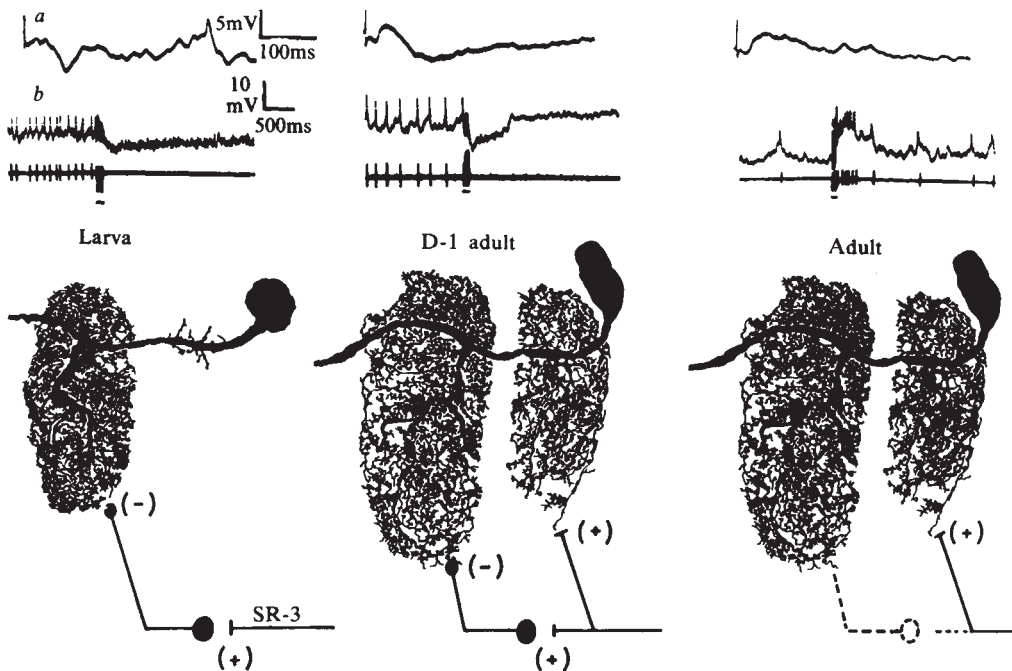


Fig. 3 Model for the changes observed in the interaction between MN-1 and SR-3. *a*, Response of MN-1 to single stimuli delivered to the contralateral SR-3. *b*, Response of MN-1 to a burst of stimuli (100 Hz) to the contralateral SR-3, which mimics the response of the receptor to natural stretch. Intracellular (top) and extracellular (bottom) records from MN-1. In the larva MN-1 is inhibited by the contralateral SR-3 through a polysynaptic pathway. The processes of SR-3 do not cross the midline of the ganglion, and lie in a different region of neuropil from the main neurite of MN-1. Single stimuli evoked long-latency i.p.s.p.s. On the day before adult emergence (D-1 adult) SR-3 evoked a biphasic response in the contralateral MN-1; the original inhibitory path was co-activated with a new direct excitatory path. Bursts of receptor spikes, however, still inhibited MN-1. The new dendritic processes of MN-1 overlapped with those of SR-3. After adult emergence, the inhibitory component was lost, and the SR-3 evoked a pure depolarizing response in the contralateral MN-1.

The inhibitory interaction between SR-3 and MN-1 is permanently inactivated after adult emergence. However, a reversible inactivation of parallel inhibition might be applicable to other situations in which hormones or other factors alter markedly the function of a particular neural pathway.

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A transient outward current in a mammalian central neurone blocked by 4-aminopyridine

B. Gustafsson*, M. Galvan†, P. Graf† & H. Wigström*

* Department of Physiology, University of Göteborg, PO Box 33031, S-400 33 Göteborg, Sweden

† Department of Physiology, University of Munich, Pettenkoferstrasse 12, 8000 Munich 2, FRG

It is becoming increasingly clear that nerve cells in the mammalian central nervous system (CNS) have a very complex electroresponsiveness. They exhibit not only time- and voltage-dependent Na^+ and K^+ conductances, analogous to those in the squid giant axon¹, but also a variety of other conductances that have a significant role in the control of cell excitability. Of the outward currents, there are, in addition to the delayed rectifier, the Ca^{2+} -activated K^+ current^{2,3} which underlies the long-lasting spike afterhyperpolarization, and the M current⁴, a non-inactivating K^+ current evoked by membrane depolarization and blocked by muscarinic, cholinergic agonists. We demonstrate here the existence in a mammalian central neurone (hippocampal CA3 pyramidal cells) of yet another outward current, which is transient and may be carried by K^+ ions. Further, the experiments show that this current is substantially reduced by the convulsant 4-aminopyridine (4-AP)⁵, resulting in a marked increase in cell excitability.

The experiments were performed on transverse slices (400–600 μm thick) of guinea pig hippocampus. The slices were kept either half or totally immersed (see below) in an experimental chamber perfused with a solution containing (in mM): NaCl, 123; KCl, 3.0; CaCl_2 , 2.0; MgSO_4 , 2.0; NaHCO_3 , 26; glucose, 10. The temperature was kept constant at either 33 °C or 26 °C. Neurones in the CA3 region were impaled with 3 M KCl-filled microelectrodes and voltage-clamped using a single electrode switched clamp circuit (a DAGAN 8100 or a custom-built amplifier of similar design⁶); the switching frequency was 3 kHz and the duty cycle was 10–25%. Possible artefacts involved in the use of these instruments have been discussed elsewhere⁷. Drugs were applied in fixed concentrations to the bathing solution and the data collected were either computer-averaged or photographed from the oscilloscope screen using Polaroid film.

Figure 1a illustrates that in control solution, constant-current pulses applied to CA3 neurones at a resting potential of -70 mV evoked electrotonic potentials (ETPs) which were smaller in the depolarizing than in the hyperpolarizing direction. Closer examination of these depolarizing ETPs shows that they increased rapidly, then suddenly levelled off in a manner which suggests that an opposing outward current had been activated. This behaviour was more clearly seen when Na^+ and Ca^{2+} action potentials were blocked by addition of tetrodotoxin (TTX) and Mn^{2+} ions (Fig. 1b). A similar outward-going rectification (albeit at more depolarized membrane potentials) has been observed in sympathetic and hippocampal CA1 neurones and attributed to depolarization-activation of M channels^{4,8,9}. However, addition of muscarine in sufficient concentrations to block M channels, did not noticeably affect the observed outward rectification (Fig. 1c). Thus it seems that, in these

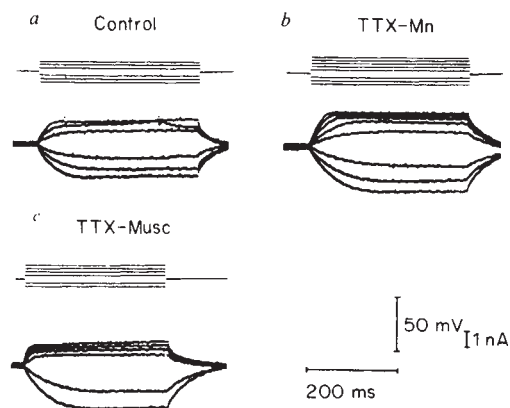


Fig. 1 Recordings of electrotonic potentials (ETPs) in CA3 neurones at 26 °C. *a*, Control solution; *b*, in solution containing 3 μ M TTX and 4 mM $MnCl_2$; *c*, in solution containing 1 μ M TTX and 25 μ M muscarine chloride. (Slices were kept totally immersed in the solutions.) The membrane potential was set at -70 mV by passing steady d.c. current through the recording microelectrode, and the amplifier was set in the switched (sample-and-hold) current clamp mode. The top traces show rectangular constant current pulses and the lower traces the resulting membrane potential deflections. *a* and *b* were recorded from the same cell and illustrate the marked attenuation of the amplitude of depolarizing ETPs. Note the slow depolarizing 'creep' of the second largest depolarizing ETP leading to an action potential towards the end of the pulse. This creep was also often observed in the presence of inward current blockers. As judged from the amplitude of the smallest depolarizing and hyperpolarizing pulses, membrane resistance increased in the TTX-Mn solution, possibly due to blockade of tonic synaptic activity. *c* was recorded from another neurone and illustrates that the outward rectification was still present after blockade of possible M-currents by muscarine (Musc).

neurones, there is an outward rectifying current operating in the threshold range, that is distinct from M-channel activation.

Figure 2 shows voltage-clamp recordings from a CA3 neurone in a solution containing TTX. From a holding potential of -70 mV (Fig. 2*a*), hyperpolarizing voltages elicited small inward currents, which were relatively linear and time independent. When the cell was depolarized to potentials greater than -60 mV, a rapidly activating outward current was observed, which decayed over the next several hundred milliseconds. The peak amplitude of this transient outward current was increased as the cell was stepped to more positive potentials. This current-

voltage relationship is plotted in Fig. 2*c* (closed circles); it is nonlinear, indicative of voltage-dependent activation. It was also observed that the current amplitude varied with the holding potential, increasing with membrane hyperpolarization (-70 to -100 mV) and decreasing to zero at holding potentials more positive than -55 mV. Thus, when the same cell was held at -40 mV and subjected to step hyperpolarizations, only steps to potentials more negative than -55 mV were followed, on return to the holding potential, by a transient outward current (Fig. 2*b*). The relationship between the hyperpolarizing step and the resulting outward current is shown in Fig. 2*c* (open circles) and is an approximate measure of the inactivation characteristic of this current.

The transient outward current was observed in all CA3 neurones examined ($n = 35$), and had an amplitude of 4.2 ± 1.5 ($\pm$ s.d.) nA ($n = 9$) as measured 30 ms following a 50–55 mV, 1–1.5 s hyperpolarizing voltage step from -30 mV, and back again. The activation kinetics were too rapid to be resolved using the single electrode clamp technique; our data show only that the current appears to have peaked within 10–15 ms. The inactivation consisted of an initial rapid phase followed by a very slow second phase, which lasted for up to several seconds (Fig. 2*b*); this form of decay could not be described by first-order kinetics.

A transient current having these characteristics has been described in invertebrate^{10,11} and vertebrate¹² neurones and has been shown to be mainly carried by K^+ ions. In the present experiments, an increase in the external K^+ ion concentration (40–60 mM with totally immersed slices) always decreased or abolished the transient outward current. A clear reversal was not obtained but this may simply reflect poor diffusion of K^+ ions into the slice, resulting in only a small positive shift of the K^+ reversal potential. Outward rectification, probably caused by activation of the transient outward current, persisted in solutions containing Ca^{2+} -channel blockers (Mn^{2+} ions) (Fig. 1*b*). Under voltage-clamp, it was also observed that the current was not blocked by Mn^{2+} , although the activation-voltage curve seemed to be shifted ~ 10 mV in the positive direction. This is, to a lesser extent, evident also from Fig. 1*b*. Thus, the results demonstrate the presence of a transient outward current in CA3 neurones, which induces a marked rectification in the current-voltage relation at membrane potentials close to threshold. This current is probably a K^+ -current that is not dependent on Ca^{2+} ion movements.

It has been shown previously that the convulsant agent 4-AP can (at mM concentrations) selectively reduce the amplitude of transient outward currents in invertebrate neurones¹³ as well

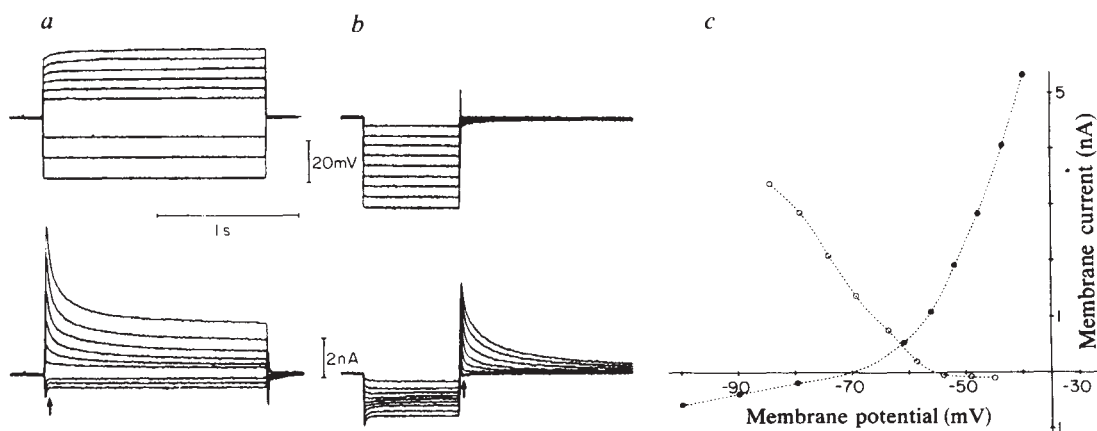
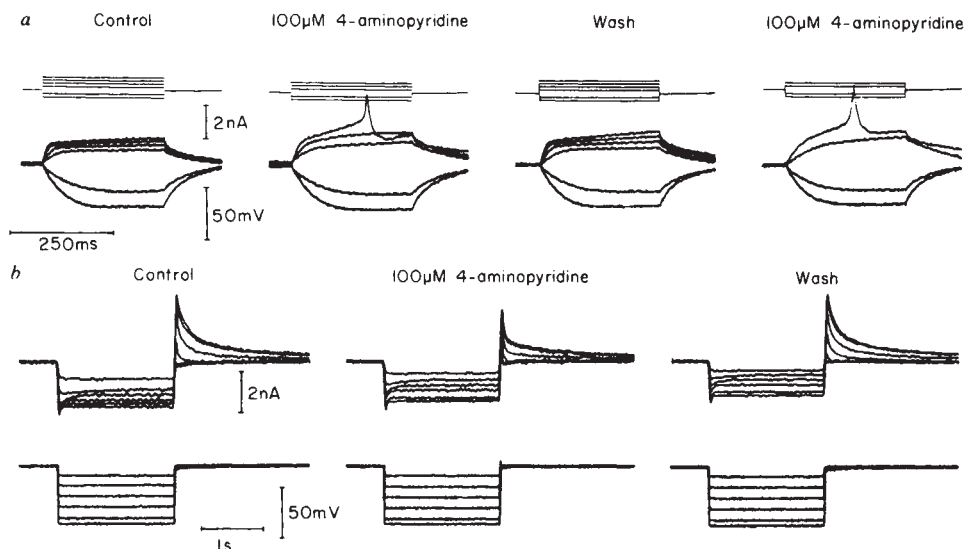


Fig. 2 Computer-averaged recordings of membrane voltage (upper traces) and current (lower traces) from a single CA3 neurone held at -70 mV (*a*) and -40 mV (*b*). Temperature, 33 °C. Slices were kept half-immersed in the solutions. Transient outward currents were elicited by step depolarizations from -70 mV or following hyperpolarizations from -40 mV. Note that for large pulses, the membrane voltage attained initially deviated from the command level. This results from inadequate feedback gain—a consequence of single electrode clamping with relatively high resistance microelectrodes. In *b*, small inward current relaxations can be observed during small hyperpolarizing commands, suggesting the presence of M-current. Measurements of membrane voltage and current, made at the times indicated by the arrows in *a* and *b*, respectively, are plotted in *c*. ●, The current-voltage curve from -70 mV; ○, the relationship between hyperpolarizing command potential and the resulting post-pulse current.

Fig. 3 Effect of 4-AP on electrotonic potentials (*a*) and transient outward currents (*b*) measured in two separate CA3 neurones. The experiment illustrated in *a* (26°C) was performed in the presence of 1 μ M TTX and 25 μ M muscarine chloride; *b* (33°C) was performed in 0.5 μ M TTX. The slices in *a* were kept totally immersed, while those in *b* were kept half-immersed. In *a* the membrane potential was manually set at -70 mV; in *b* the holding potential was -30 mV. The current clamp records in *a* show electrotonic potentials before, at the end of and 60 min after bath application (8 min) of 100 μ M 4-AP. After a substantial recovery had been obtained, 4-AP was applied again for a longer period (20 min). Note that a maximum effect was not achieved during the first application, as evidenced by the much larger change from outward rectification resulting from the second, longer application. The amplitude of hyperpolarizing ETPs was almost unaltered by 4-AP as was the current necessary to hold the membrane at -70 mV. In *b*, the voltage clamp records illustrate that a brief (5 min) application of 100 μ M 4-AP substantially and reversibly reduced transient outward currents.



as in mammalian cardiac muscle fibres¹⁴. Figure 3*a* demonstrates that in CA3 pyramidal cells, 100 μ M 4-AP can reversibly reduce the outward rectification, resulting in a considerable increase in cell excitability; evidence for this comes from the appearance of a Ca^{2+} action potential. Furthermore, Fig. 3*b* shows that in voltage-clamp, the transient outward current was substantially blocked by 4-AP. These effects were evident after only a few minutes of 4-AP application at times when it was unlikely that the 4-AP concentration equalled that in the bathing solution. The classical K^{+} -channel blocker tetraethylammonium chloride (TEA), which blocks delayed rectifier channels¹⁵ and Ca^{2+} -activated K^{+} channels in vertebrate neurones¹⁶, did not (at 3 mM) have any significant effect on this current. Note, however, that even in the presence of high concentrations (500 μ M) of 4-AP (in which no transient outward currents were observed), some outward rectification from -70 mV remained. This may be related to the presence of M currents, which were also observed in these cells.

Thus, hippocampal CA3 pyramidal cells exhibit a transient outward current distinct from the delayed rectifier K^{+} current, the Ca^{2+} -activated K^{+} current and the M current. The activation-inactivation characteristics as well as the pharmacology are similar, in principle, to those of the so-called A current in invertebrate neurones and it seems reasonable to extend this terminology to mammalian central neurones. We cannot, however, be certain that the current described here is similar in all respects to that of invertebrates. Connor and Stevens¹⁷ proposed that the A current controls repetitive firing in gastropod neurones. Its long duration and large amplitude in CA3 neurones also suggest that A currents in these cells, together with the Ca^{2+} -activated K^{+} conductance, could serve such a role. More importantly, our data show that A currents are capable of controlling the approach of membrane potential to spike threshold and thus contribute to the control of cell excitability. The activation kinetics suggest that A currents may even affect the action potential (see ref. 18) and excitatory postsynaptic potential trajectories. The postsynaptic excitatory effect of 4-AP can probably be explained by blockade of A currents, although other contributory mechanisms cannot yet be excluded¹⁹. The presence of such currents also in vertebrate nerve terminals might explain the facilitatory actions of aminopyridines on neurotransmitter release¹⁹. Nevertheless, it certainly seems that the postsynaptic effects of 4-AP may contribute to its convulsant action.

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Serotonergic denervation partially protects rat striatum from kainic acid toxicity

Michael Berger, Günther Sperk & Oleh Hornykiewicz

Institute of Biochemical Pharmacology, University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria

We report here observations indicating that the rat striatum can be partially protected from the neurotoxic action of locally applied kainic acid (KA, a rigid structural analogue of glutamic acid¹) by procedures resulting in reduced serotonergic brain activity. This observation may be important because the striatal damage produced by KA shows morphological and neurochemical similarities to the changes seen in the striata of patients dying of Huntington's chorea^{2,3}, an hereditary brain disorder, and because functional and morphological brain changes induced by KA have been proposed as a close model for human epilepsy⁴. Our observations thus suggest that serotonergic brain mechanisms influence the development of neuropathological changes accompanying certain neurological disorders.

Half-maximal lesion of the striatum (with respect to marker enzymes for acetylcholine and γ -aminobutyric acid neurones, see Fig. 1b; for enzyme assays, see Fig. 1 legend) was reproducibly obtained by applying stereotactically (David Kopf apparatus) 2 nmol of KA dissolved in 1 μ l 0.9% buffered saline into the rostral part of the striatum (coordinates A: 7.9, L: 2.5, D: 0.9, according to the rat brain atlas of König and Klippel⁵) at a rate of 0.33 μ l min⁻¹. For all experiments male albino rats (Sprague-Dawley; Versuchstierzuchtanstalt Himberg, Austria) weighing 250–350 g were used. For stereotaxic operations the animals were anaesthetized with 50 mg per kg intraperitoneal (i.p.) pentobarbital; intracisternal (i.c.) injections were performed under ether anaesthesia.

The experimental results are expressed as percentage of enzyme activity related to the contralateral striatum (into which no KA was injected). This strategy seemed justified as the various drug pretreatments, which always affected both striata, had no statistically significant effect on the absolute enzyme activities, and the striatal KA-induced lesion did not influence the enzyme levels in the contralateral striatum. The use of each animal as its own control permitted the detection of even small drug effects on the KA-induced changes. In this respect, direct comparison between the means of absolute enzyme activities of the different groups yielded identical results; however, due to inter-individual variation, the degree of statistical significance between the groups was, as expected, correspondingly smaller.

As shown in Fig. 1a, administration of 200 μ g 5,7-dihydroxytryptamine (5,7-DHT) into the cisterna magna (i.c.)⁶ 10 days before intrastratial microinjection of 2 nmol KA, reduced the effect of KA on the activity of the striatal marker enzymes glutamic acid decarboxylase (GAD) and choline acetyltransferase (CAT). The difference between the group of animals receiving KA alone and that pretreated with 5,7-DHT was statistically significant at the 0.1 per cent level (Student's *t*-test). The striatal serotonin level in the 5,7-DHT-injected animals was reduced to 40% of control value.

The same degree of protection of the striatum from KA toxicity as that attained by 5,7-DHT, could be achieved

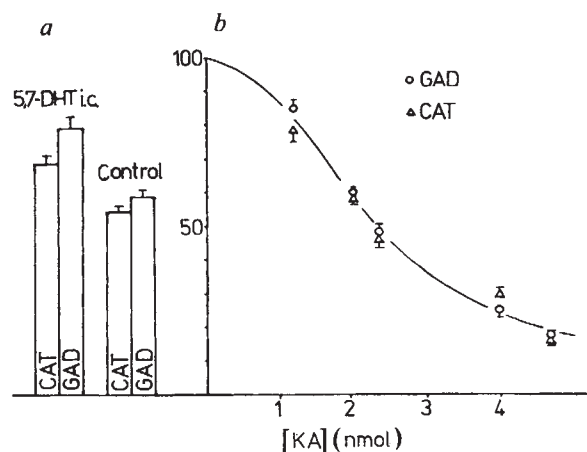


Fig. 1 Protective effect of i.c. injections of 5,7-dihydroxytryptamine (5,7-DHT) against the striatal toxicity of 2 nmol kainic acid (KA) (a), compared with the dose dependency of striatal KA-induced lesions (b). Activities of the striatal marker enzymes, measured 7 days after KA injection, are indicated on the ordinate as percentage of the contralateral non-injected striatum (means $\pm$ s.e.m.). Glutamic acid decarboxylase (GAD) was assayed by trapping ¹⁴CO₂ formed from radiolabelled glutamic acid following the procedure of Miller *et al.*¹⁹. L-[1-¹⁴C]glutamic acid (4 nM), 0.5 mM pyridoxal phosphate and 1 h incubation at 37 °C were used. Choline acetyltransferase (CAT) was assayed by the Fonnum method²⁰ using [1-¹⁴C]acetyl-CoA (0.2 mM), 8 mM choline chloride, 0.1 mM physostigmine and 30 min incubation at 37 °C. ¹⁴C-acetylcholine was extracted with heptanone (3) containing 1% Na-tetraphenylborate. In the 5,7-DHT-treated rats striatal levels of serotonin were reduced by 60%, whereas dopamine levels remained unchanged, as determined by HPLC with electrochemical detection^{15,21}.

Table 1 Effect of manipulations influencing brain serotonin on striatum lesioned with 2 mol kainic acid (injected intrastratially)

	% CAT $\pm$ s.e.m.	(n)	% GAD $\pm$ s.e.m.	(n)
5, 7-DHT i.c. + KA	68.9 $\pm$ 2.4	(14)*	79.3 $\pm$ 3.7	(14)*
KA	54.7 $\pm$ 1.7	(15)	59.0 $\pm$ 2.0	(15)
p-Cl-Phe i.p. + KA	74.0 $\pm$ 6.4	(4)†	79.9 $\pm$ 5.1	(4)†
KA	57.0 $\pm$ 4.0	(6)	59.8 $\pm$ 4.4	(6)
Mergoline i.p.				
+ KA	70.4 $\pm$ 1.9	(6)†	70.6 $\pm$ 2.6	(6)†
Solvent + KA	59.1 $\pm$ 3.6	(7)	58.7 $\pm$ 4.4	(7)
5,7-DHT i.str.				
+ KA	71.4 $\pm$ 3.2	(8)†	73.9 $\pm$ 4.0	(7)†
Saline + KA	62.0 $\pm$ 2.6	(7)	59.0 $\pm$ 4.5	(7)
Pargyline + 5-OH-Trp i.p. + KA	62.3 $\pm$ 3.9	(15)	65.0 $\pm$ 2.9	(15)
KA	57.3 $\pm$ 2.7	(12)	58.2 $\pm$ 3.0	(12)
5-MDMT + KA	58.2 $\pm$ 5.9	(7)	65.3 $\pm$ 5.0	(7)
KA	61.1 $\pm$ 3.4	(8)	62.8 $\pm$ 3.4	(8)

5,7-Dihydroxytryptamine (5,7-DHT) was administered intracisternally (i.c.) or intrastratially (i.str.) in N₂-bubbled 0.9% NaCl containing 0.05% ascorbic acid, 10 days before kainic acid (KA); the rats were pretreated with desipramine (25 mg per kg, i.p.), controls to i.str. 5,7-DHT injections received i.str. saline. p-Chlorophenylalanine (p-Cl-Phe; 300 mg per kg, in saline) was injected i.p. 6 days before KA. Mergoline (12 mg per kg) was dissolved in 5% polysorbate 80 and injected i.p. 1 h before KA; controls received 5% polysorbate 80 i.p. Pargyline (25 mg per kg, in saline) and L-5-hydroxytryptophan (5-OH-Trp; 5 mg per kg, in saline neutralized with acetic acid) were injected i.p. 30 and 15 min, respectively, before KA, and 5-methoxy-N,N-dimethyltryptamine (5-MDMT; 3 mg per kg, in saline) 15 min before KA. Only animals showing typical 'serotonin related symptoms'¹⁸ (especially 'wet dog shakes') were used for KA injection. Values are means $\pm$ s.e.m., expressed as percentage of enzyme activity related to the contralateral non-injected striatum. Ranges of enzyme activities in the contralateral striata were for CAT, 24–38 nmol ¹⁴C-acetylcholine and for GAD, 11–16 nmol ¹⁴CO₂ formed per and mg wet tissue weight. Significance of the difference between corresponding groups was calculated using Student's *t*-test. Number of animals is given in parentheses.

* *P* < 0.001; † *P* < 0.05 compared with controls.

by i.p. pretreatment with p-chlorophenylalanine (p-Cl-Phe, 300 mg per kg), a serotonin-depleting agent⁷, or mergoline (12 mg per kg), a serotonin receptor blocker⁸ (Table 1). Although the protective effect of these drugs was of the same magnitude as that of 5,7-DHT, the statistical significance of the achieved differences was lower (0.02 < *P* < 0.05). However, taken together, these results strengthen the conclusion that the protective effect of i.c. 5,7-DHT against the striatal toxicity of KA was based on a reduction of serotonin levels in the brain.

Because 5,7-DHT given i.c. reduced not only the striatal serotonin but also the serotonin in the thalamus (–75%) and frontal cortex (–90%) (both brain regions are major sources of striatal input neurones), we tested the striatal specificity of the protective effect of 5,7-DHT. Thus, in a separate experiment, injection of 5 μ g 5,7-DHT (dissolved in 1 μ l 0.9% saline) bilaterally into the medial part of the striatum (coordinates⁵ A: 6.8, L: 3.6, V: 0.2) reduced the striatal serotonin levels by 65% whereas those in the frontal cortex were reduced by only 35%, with the thalamic serotonin remaining unchanged. Dopamine levels in the striatum were moderately reduced (–35%). The intrastratial 5,7-DHT pretreatment, similar to the i.c. application, again afforded the same degree of protection against striatal KA toxicity, as judged by striatal GAD and CAT activities (Table 1). This indicates that the serotonergic system of the striatum itself was involved in the observed 5,7-DHT effect.

On the other hand, we have been unable to render intrastratial KA more neurotoxic by i.p. pretreatment of the rat with pargyline plus 5-hydroxytryptophan (5-OH-Trp) or 5-methoxy-N,N-dimethyltryptamine (5-MDMT, Table 1), although both procedures can be expected to increase brain serotonin activity. This suggests that serotonin may influence

KA toxicity indirectly, especially as the correlation between kainate-induced neuronal degeneration and 5,7-DHT-induced striatal serotonin depletion was only moderately significant ($r = 0.50$ with 20 degrees of freedom, $0.02 < P < 0.05$).

In search of an explanation for our results, it is interesting that iontophoretically applied serotonin is generally assumed to exert an inhibitory influence on striatal neurone activity⁹⁻¹¹. Cortical ablation, which interrupts the excitatory glutamatergic input to the striatum, results in a dramatic reduction of striatal KA toxicity¹², so that we cannot exclude the possibility that deafferentation of the striatum as such may exert some protective effect, regardless of whether the transmitter involved is excitatory (as the cortico-striatal glutamatergic input is supposed to be) or not. However, in contrast to iontophoretically applied serotonin, the synaptically released amine may indeed have a net excitatory effect on striatal neurones. This is indicated by observations showing that lesion of the raphe-striatal serotonin pathway results in a substantial reduction of acetylcholine turnover in the striatum¹³. In fact, our present results, seen in conjunction with the effect of interruption of the glutamatergic cortical input to the striatum, may be taken

as indirect support for an excitatory role of striatal serotonin. Regardless of the nature of the neurophysiological effect of striatal serotonin, our results strengthen the notion that KA, in addition to its direct excitotoxic activity, triggers circuits of neuronal hyperactivity including the area injected and some particular afferents as essential components¹⁴. The dramatic rise in striatal serotonin (and dopamine) turnover observed after local injection of KA^{15,16} could reflect the involvement of these afferents in KA-elicited hyperactivity circuits. In this respect, it may be noteworthy that in Huntington's disease the severe loss of the intrinsic and output neurones population of the affected striatum results in a relative predominance of striatal input systems including those containing serotonin and dopamine¹⁷. It would be interesting to know if this relative and progressive predominance of striatal serotonin and dopamine exerts any detrimental influence on the time course of the morphological and functional deterioration of the Huntington striatum.

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Bradykinin receptor-mediated chloride secretion in intestinal function

Donald C. Manning & Solomon H. Snyder*

Departments of Neuroscience, Pharmacology and Experimental Therapeutics, Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205, USA

James F. Kachur, Richard J. Miller & Michael Field

Departments of Pharmacological and Physiological Sciences and Medicine, University of Chicago, Chicago, Illinois 60637, USA

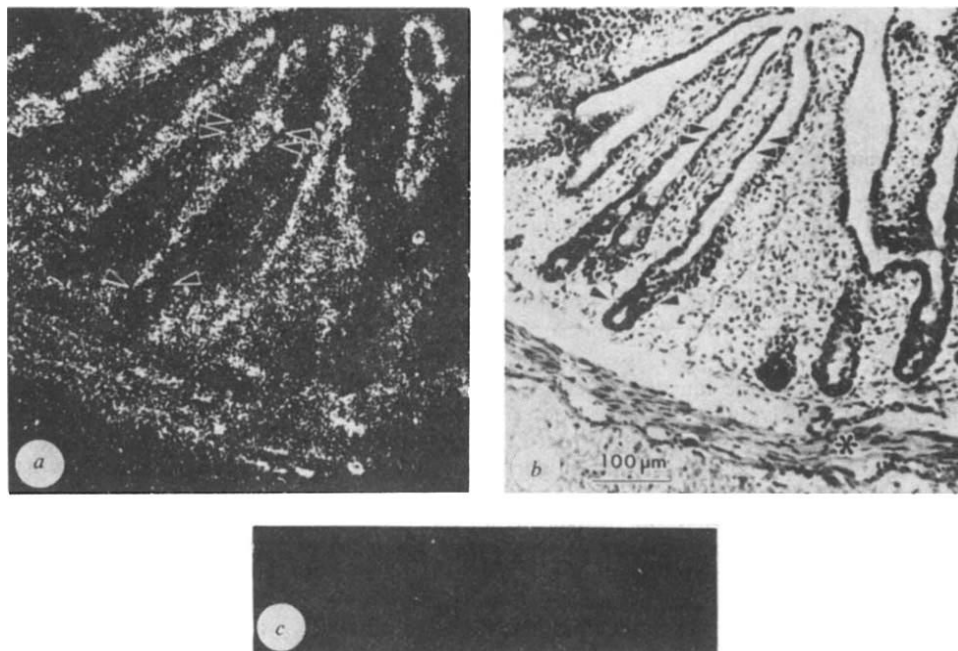
The nonapeptide bradykinin (Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and the decapeptide kallidin (Lys-bradykinin) are released during tissue damage and cause vasodilation, increased vascular permeability, altered gut motility and pain¹⁻⁴. Accordingly, they have been implicated in several pathological processes, including carcinoid tumours and postgastrectomy dumping syndrome. Symptoms of these two syndromes include flushing, light-headedness, headaches and diarrhoea⁵⁻⁹. Increased blood levels of bradykinin^{5,7-9} and kallikrein⁹, the bradykinin-releasing enzyme, have been noted in such patients and may explain these symptoms. However, while kinin-forming and -destroying activity has been noted in the gut¹⁰⁻¹⁴, bradykinin itself has not been directly demonstrated. Moreover, while bradykinin influences gut motility and electrical activity^{15,25,26}, most clinical diarrhoea is due to ion and fluid secretion into the gut lumen rather than altered motility¹⁶. We describe here specific bradykinin receptors in intestinal mucosa and muscle. We also report that bradykinin and several peptide analogues potentially stimulate chloride secretion in the gut with a peptide specificity indicative of specific receptor interactions. These findings suggest a physiological and a pathological role for kinins in intestinal function. Throughout this report the term kinin will indicate both bradykinin and kallidin activities since in several cases the true active form is unknown.

* To whom correspondence should be addressed.

Previously, we demonstrated specific ³H-bradykinin receptor-binding sites in guinea pig ileum¹⁷. The potencies of peptides at these binding sites correlated with their potencies in contracting smooth muscle, suggesting that the binding sites might be localized to muscle. The results shown in Table 1 demonstrate that specific bradykinin receptor-binding sites occur in the mucosa as well as in the muscle area. The relative potencies of bradykinin and six analogues listed in Table 1 are quite similar in both mucosa and muscle and correspond well with those observed previously in the total ileal tissue¹⁷. The 25-fold-enhanced potencies of the peptides observed in this study, compared to our earlier work, are due to enhanced specific activity and diminished degradation of the ³H-bradykinin. The relative potencies of bradykinin and its analogues at the ³H-bradykinin receptor-binding site closely parallels their potency in stimulating active chloride secretion. Removal of the arginine in the 9 position, forming Des-Arg⁹-bradykinin, destroys receptor-binding affinity and eliminates biological activity. Replacement of proline in position 3 with α -aminoisobutyric acid (AIB), forming AIB³-bradykinin reduces receptor-binding potency markedly in parallel with a decrease in biological activity. In contrast to internal changes in the bradykinin structure, addition of lysine to the amino-terminal only reduces the receptor-binding and secretory activity slightly. While Met-Lys-bradykinin is 30-50% as potent as bradykinin in affecting short-circuit current, it is only 10% as potent at ³H-bradykinin receptor-binding sites. It is possible that in the short-circuit current assay, Met-Lys-bradykinin is metabolized to the more potent Lys-bradykinin while conditions of the binding assay retard such metabolism (see Table 1 legend).

The localization of bradykinin receptors to both the mucosa and muscle layers of the ileum was confirmed by light microscopic autoradiographic studies (Fig. 1). Autoradiographic grains were apparent in highest density over the lamina propria surrounding both villus and crypt epithelial cells. Grains were also concentrated over the circular and longitudinal muscle layers. By contrast, grain density was sparse or absent over lymphatic tissue, as well as the myenteric ganglia. Inclusion of 1 μ M unlabelled bradykinin in incubations greatly reduced

Fig. 1 Autoradiographic localization of ^3H -bradykinin (BK) receptor binding in guinea pig ileum. The dark-field photomicrograph (a) shows the autoradiographic grain distribution over the same tissue as the bright-field photomicrograph (b). Note that grains are concentrated over muscle layers (*) and also over the villi (between double arrows). Grains are almost entirely localized over the centre or lamina propria of the villi and are sparse or absent over the luminal surface of the epithelium. Grains are also absent inside the crypts of Lieberkuhn (single arrows) but are concentrated over the serosal side of the crypt epithelial cells. The dark-field photomicrograph (c) shows the absence of specific receptor binding in an adjacent 10- μM section when 1 μM unlabelled BK was included in the incubation buffer. The coverslip method of Young and Kuhar³³ was used. Guinea pigs were perfused through the heart with 500 ml of 0.1% formaldehyde in a mixture of equal parts of phosphate-buffered saline and 20% sucrose, pH 7.4. The ilea were then removed, embedded in homogenized calf cerebral cortex and flash-frozen in liquid nitrogen. Sections (10 μM thick) were cut in a cryostat at -12°C , thaw-mounted onto chrome alum gelatin subbed microscope slides and stored frozen at -20°C . Slides were warmed to room temperature, then incubated for 120 min at 4°C in medium containing 25 mM trimethylaminoethanesulphonic acid (TES) pH 6.8, 1 μM 1,10-phenanthroline, 0.2% bovine serum albumin (BSA) (protease-free), 1 mM dithiothreitol, 0.1 mM bacitracin, 1 μM captopril (SQ 14,225), 300 mM sucrose, and 0.2 nM ^3H -BK (52 Ci mmol^{-1}). Blanks were incubated in the same medium with the addition of 1 μM unlabelled BK. After incubation tissue sections were washed in 25 mM TES pH 6.8, and 1 mM 1,10-phenanthroline for 60 min (2×30 min) at 4°C . Sections were then dried rapidly in cold, dry air, NTB-2 emulsion-coated coverslips were apposed and the sections were exposed to the emulsion for 1 month.



grain density with the remaining grains being distributed diffusely. The pharmacological specificity was also indicated by biochemical experiments using the six bradykinin analogues listed in Table 1 and slide-mounted tissue sections. After incubation and washing, the tissue section was wiped off the slide with a circle of filter paper and the bound radioactivity assessed

by scintillation counting. In these experiments the affinity of bradykinin receptors was the same as in homogenate estimations. Also, ^3H -bradykinin binding is displaced by peptides with the same potencies as in homogenate experiments.

Bradykinin receptors in the muscle layers presumably mediate the contractile effects of bradykinin. To investigate the

Table 1 Relative potencies of bradykinin (BK) and several related peptides in receptor binding and short-circuit current assays

Kinin	Structure	Stimulation of short-circuit current $\text{EC}_{50}(\text{nM})$	Inhibition of ^3H -BK binding	
			Mucosa $\text{IC}_{50}(\text{nM})$	Muscle layer $\text{IC}_{50}(\text{nM})$
	1 2 3 4 5 6 7 8 9			
Bradykinin (BK)	H-Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH	1.0 ± 0.2	0.2	0.2
Kallidin (Lys-BK)	Lys-Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH	2.8 ± 0.5	0.4	0.2
Met-Lys-GK	Met-Lys-Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH	2.6 ± 0.8	1.8	2.2
AIB ³ -BK		183.0 ± 44	5.0	5.0
Hydroxyproline ³ -BK		3.1 ± 0.3	0.4	0.6
β -(2-Thienyl)-Ala ^{5,8} -BK		5.8 ± 1.4	0.5	0.4
Des-Arg ⁹ -BK		No response	$>10^{-6}$	$>10^{-6}$

^3H -BK was prepared (by NEN) by reduction of 2,3-dehydroproline-BK with tritium gas, resulting in a specific activity of either 27 or 52 Ci mmol^{-1} . The radiochemical purity of the ligand was determined by HPLC both before and after binding assay. The HPLC system used separates all partial sequence fragments of BK including BK₂₋₉, BK₁₋₈ and BK₁₋₇. A $\mu\text{Bondapak C}_{18}$ (Waters Ass.) column (4 mm i.d. $\times$ 30 cm) was used with a linear gradient solvent system (a, $\text{H}_2\text{O} + 0.2\% \text{H}_3\text{PO}_4$, pH 2.4; b, $\text{CH}_3\text{CN} + 0.2\% \text{H}_3\text{PO}_4$; 12% B $\rightarrow$ 24% B) over 20 min with a flow rate of 2.0 ml min^{-1} . Fractions (30-s) were collected and the radioactivity measured by liquid scintillation counting. Using this HPLC system we were able to monitor the metabolism of the ^3H -ligand after the binding assay and thus develop a mixture of peptidase inhibitors effective in stabilizing BK. This increase in BK stability and use of a ligand of 2–4 times higher specific activity accounts for the almost 10-fold greater detected affinity of BK for its receptor than published previously¹⁷. In all assays mucosa was separated from the muscle layers by scraping gently with a glass slide. Crude membranes were prepared by homogenizing tissues in 20 vol 25 mM TES, pH 6.8 + 1 mM 1,10-phenanthroline with a Brinkmann Polytron PT10 (setting 6 for 30 s). The homogenates were centrifuged twice at 50,000g for 10 min with an intermediate rehomogenization in buffer. For routine studies the final pellets were resuspended in 300 vol of incubation buffer (25 mM TES pH 6.8, 1 mM 1,10 phenanthroline, 0.2% BSA (Sigma; crystallized, lyophilized), 0.1 mM bacitracin, 1 mM dithiothreitol, 1 μM captopril). To triplicate polypropylene incubation tubes were added (in a total volume of 4 ml); 850 μl of freshly resuspended tissue, 25,000 d.p.m. of ^3H -BK (final concentration 0.05 nM) and competing agents. After incubation for 90 min at 4°C the samples were rapidly filtered over GF/B filters previously coated with 0.1% aqueous polyethyleneimine solution, then the filters were washed three times with 3 ml ice-cold buffer. Filter-bound radioactivity was determined by liquid scintillation counting at 40% efficiency in 10 ml Formula 947 (NEN). Typical c.p.m. were 1,000 total and 35 blank with a tissue concentration of 1 ml tissue wet weight per ml. For metabolism studies, samples were centrifuged after incubation and the supernatant analysed by HPLC. E_{max} , maximal effect; EC_{50} , concentration to elicit 50% maximal effect relative to bradykinin E_{max} in stimulating I_{sc} in guinea pig ileum; IC_{50} , concentration of nonradioactive BK or BK analogue required to displace 50% of specifically bound ^3H -BK from its receptor EC_{50} values represent mean $\pm$ s.e.m.

Table 2 Effect of bradykinin on chloride fluxes

	Cl ⁻ flux		Net
	m → s	s → m	
Control	8.2 ± 0.8	8.7 ± 0.8	-0.5 ± 1.6
Bradykinin (10 ⁻⁵ M)	8.3 ± 0.4	12.1 ± 1.1*	-3.8 ± 1.3*

Values are the μ equiv. $\text{cm}^{-2} \text{h}^{-1} \pm \text{s.e.m.}$ for six paired experiments. Two 10-min samples were taken starting 15–20 min after the addition of $^{36}\text{Cl}^-$ and 5 min after adding bradykinin. * $P < 0.025$. Fluxes were measured with tissues short-circuited as described previously¹⁸. Flux is from mucosa to serosa (m → s) or serosa to mucosa (s → m).

possible function of the mucosal receptors, we evaluated the influence of bradykinin on the transepithelial potential difference (p.d.) and short-circuit current (I_{sc}) of the mucosal layer of the guinea pig ileum using techniques previously described¹⁸ (Fig. 2, Table 1). Tissue conductance showed no appreciable change and therefore the change in I_{sc} was proportional to the change in p.d. There was no effect on p.d. or I_{sc} when bradykinin in concentrations up to 10 μM was added to the mucosal bathing solution. In striking contrast, addition of nanomolar concentrations of bradykinin to the serosal surface rapidly increased p.d. and I_{sc} , with maximal effects within 2 min (Fig. 2). The finding that bradykinin acts only when added to the serosal surface agrees with the localization of bradykinin receptors to the serosal surface of villus and crypt epithelium. These effects resemble those produced in this tissue by other peptides such as vasoactive intestinal peptide¹⁹, substance P²⁰, neurotensin²⁰ and bombesin²¹.

After quickly becoming maximal, the I_{sc} diminished with a rather slower time course similar to that observed for the 'fading' of the smooth muscle-contacting effects of bradykinin⁴. Whether it is related to tachyphylaxis or peptide metabolism is unclear. In the absence and presence of the kininase II inhibitor, captopril, 50% of the maximal response occurs at 10 and 1.0 nM bradykinin, respectively. With this 10-fold enhancement of potency, bradykinin has a similar potency in altering I_{sc} and binding to receptors (Table 1).

Several experimental results suggest that the bradykinin-induced increase in I_{sc} is due to stimulation of anion secretion. Thus, the effects of bradykinin on p.d. and I_{sc} are reduced by 86% when Cl^- and HCO_3^- are replaced by sulphate (data not shown). Other evidence derives from direct measurement of unidirectional Cl^- fluxes across the short-circuited ileal mucosa in the presence and absence of bradykinin (Table 2). In control tissues there is a mean net Cl^- secretion of $0.5 \mu\text{equiv. cm}^{-2} \text{h}^{-1}$. In the presence of bradykinin, net Cl^- secretion increases to $+3.8 \mu\text{equiv. cm}^{-2} \text{h}^{-1}$. These experiments used high concentrations of bradykinin to obtain maximal and prolonged effects.

The effects of bradykinin do not seem to be mediated by influences on neuronal conduction, as they are not affected by tetrodotoxin (Table 3); calcium movements may be involved, as verapamil, a Ca^{2+} channel blocker, markedly inhibits the influences of bradykinin on I_{sc} (Table 3). Bradykinin stimulation of I_{sc} is also completely blocked following the addition of the Ca^{2+} channel blocker CoCl_2 (1 mM). A possible role of Ca^{2+} agrees with data that Ca^{2+} regulates intestinal secretion²² and bradykinin receptor binding¹⁷.

It also seems that the effects of bradykinin on Cl^- secretion may be mediated by arachidonic acid and its metabolites. We have found that the action of bradykinin can be inhibited by a variety of agents that act as inhibitors of cyclooxygenase (for example, indomethacin) and phospholipase A_2 (for example, mepacrine). Moreover, exogenously added arachidonic acid or prostaglandin E_2 (PGE_2) are both powerful secretory stimuli and bradykinin can stimulate the *in vitro* release of PGE_2 (ref. 23). Interestingly, phospholipase A_2 , the enzyme that releases arachidonic acid from membrane phospholipids, is dependent on calcium. Conceivably, bradykinin alters the availability of

calcium to phospholipase A_2 by opening calcium channels as indicated by the inhibitory effects of Ca^{2+} channel blockers.

The present results suggest that bradykinin may have both a physiological and pathological role in the intestine. Our *in vitro* findings are consistent with the observations of Hardcastle *et al.*¹⁵ that bradykinin causes a transient increase in trans-colonic and transjejunal p.d. in the rat. Independently Cuthbert and Margolius^{24,25} have also found potent enhancement of gut chloride secretion by kallidin and have also suggested a role for prostaglandins. The bradykinin receptors in the crypts might regulate this anion secretion. Bradykinin may enhance intestinal absorption of amino acids and glucose^{25,26}, effects that could involve bradykinin receptors in the tips of the intestinal villi where such absorption takes place.

Autoradiography at the light microscopic level does not allow the precise determination of the cellular location of receptors. Bradykinin receptors in the lamina propria are probably located on the basolateral membranes of the epithelial cells, however, receptors may also occur on smooth muscle cells, blood vessels and/or sensory nerve fibres in the villi.

Excessive kinin activity might account for some pathological conditions. In carcinoid syndrome, serotonin may be responsible for some of the symptoms⁵⁻⁷. However, although serotonin does elicit diarrhoea²⁸, it causes vasoconstriction⁷ rather than the vasodilatation and the associated flushing, light-headedness and headache that typically occurs in carcinoid patients. Moreover, while some increase of blood level of serotonin occurs in certain patients with carcinoid syndrome, there is no correlation with the onset of their symptoms⁷. By contrast, increase blood levels of kinins have been detected in carcinoid patients in the course of their symptoms⁵⁻⁷.

Kinins could also play a part in inflammatory bowel disease. In these conditions the plasma levels of kinins correlate very closely with the onset of vasomotor and gastrointestinal symptoms⁶⁻⁹. As inflammation is associated with the formation of kinins²⁹, one would expect kinins to be released into the local or systemic circulation. Indeed, inflamed intestinal tissue

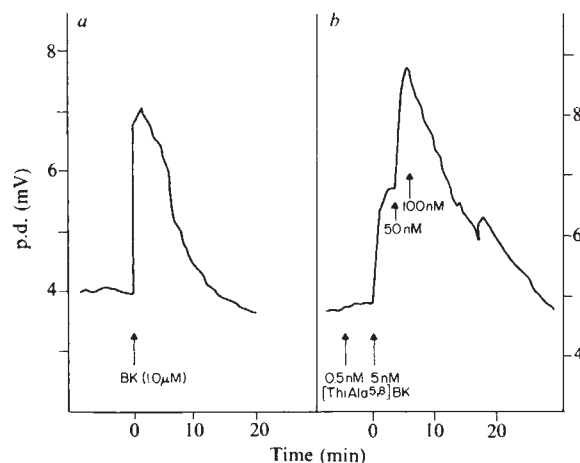


Fig. 2 Effect of BK on intestinal potential difference (p.d.). Ileal mucosa was obtained from female Hartley guinea pigs weighing 400–600 g. Segments of distal ileum, ~10–15 cm long, were excised 5 cm above the ileo-caecal junction. The segments were cut along their mesenteric border and placed in oxygenated ice-cold Ringer's. After stripping off the serosa and underlying longitudinal muscle layer, each tissue was mounted between two Leucite half chambers, the exposed areas being 0.64 cm^2 . The apparatus for measuring p.d. and I_{sc} was as described previously¹⁸. *a*, Effect of single dose of BK on transepithelial p.d. across guinea pig ileal mucosa. *b*, Effect of increasing concentrations of BK analogue (ThiAla^{5,8}) on p.d. Peptides were added at $\uparrow$. Tracings are from single experiments, but are characteristic of tracings from at least 10 other experiments.

Table 3 Effect of verapamil and tetrodotoxin on peak increments in I_{sc} elicited by bradykinin

	n	Response to 1.0 μ M bradykinin Change in I_{sc} on drug addition (μ A cm ⁻²)	(% of control response)
Tetrodotoxin	4	-44.4 $\pm$ 4.8	119.4 $\pm$ 17.1
Verapamil	4	-24.6 $\pm$ 5.2	29.9 $\pm$ 10.3*

Values are mean $\pm$ s.e.m.; n, number of paired experiments. Negative values reflect decreases in I_{sc} . * $P < 0.01$ compared with control.

from patients with ulcerative colitis contains abnormally high levels of active kallikrein, the kinin-releasing enzyme¹⁴. Plasma and tissue levels of peptidyl dipeptidase which degrades kinins, are depressed in patients with regional enteritis; this would elevate kinin levels^{30,31}. Also, in regional enteritis plasma levels of α_2 -macroglobulin are reduced³². α_2 -Macroglobulin inhibits the bradykinin releasing enzyme kallikrein, thus reduced levels of the inhibitor should facilitate kinin synthesis. If indeed kinins are associated with symptoms of carcinoid syndrome or inflammatory bowel disease, then administration of the peptidyl dipeptidase inhibitor captopril to such patients may be contraindicated.

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A new look at nonparasitized red cells of malaria-infected monkeys

C. M. Gupta, A. Alam, P. N. Mathur & G. P. Dutta

Divisions of Biophysics & Microbiology,
Central Drug Research Institute, Lucknow-226001, India

Many reports have shown that malarial parasites can produce distinct morphological and molecular alterations in the membranes of the parasitized erythrocytes¹⁻⁸, but few studies have been carried out on nonparasitized erythrocytes of infected animals⁹⁻¹¹. We report here that the outer leaflet of the membrane bilayer of nonparasitized erythrocytes contains significantly larger amounts of aminophospholipids (phosphatidylethanolamine (PE) and phosphatidylserine (PS)), than the normal red cell membrane. This alteration in nonparasitized red cells is probably caused by Ca²⁺-induced cross-linking of spectrin, and gradually disappears after chloroquine treatment. The external localization of PS in these cells together with defective structure of their cytoskeletal network provide a strong basis for the complications associated with malaria infection like thrombosis, infarction and severe anaemia.

The transbilayer distributions of the diacylglycerophospholipids, phosphatidylcholine (PC), PE and PS, in the membranes of normal erythrocytes of uninfected monkeys as well as nonparasitized red cells of *Plasmodium knowlesi*-infected monkeys were investigated using phospholipase A₂ and 2,4,6-trinitrobenzenesulphonic acid (TNBS) as external membrane probes^{12,13}. The results shown in Table 1 reveal marked differences between the two types of cells after treatments with phospholipase A₂. Unlike the normal intact cells, PE (~31%) and PS (~15%) in nonparasitized erythrocytes from infected monkeys were readily accessible to the enzyme. Almost complete (92-100%) hydrolysis of all the glycerophospholipids occurred when unsealed ghosts of the normal and nonparasitized cells were separately treated, in identical conditions, with phospholipase A₂.

Treatments of both types of cells with TNBS also reflected notable differences (Table 2). In the nonparasitized cells, TNBS

Table 1 Erythrocyte phospholipid degradation by phospholipase A₂

Sample	n*	PC (%)	PE (%)	PS (%)
Normal red cells	15	57.35 $\pm$ 4.11	0	0
Nonparasitized red cells	12	48.31 $\pm$ 4.23	31.31 $\pm$ 2.40	15.19 $\pm$ 2.32

Synchronous infections of *Plasmodium knowlesi* (WI strain) were maintained in healthy rhesus monkeys as described earlier⁸. The monkeys were bled when parasites were at the schizont stage. Blood was drawn into heparinized glass tubes and washed three times with phosphate-buffered saline (pH 7.2). Leukocytes and platelets from normal blood and leukocytes, platelets and schizonts from infected blood were removed by means of a Ficoll-Hypaque gradient²⁶. The nonparasitized red cells thus obtained were invariably contaminated with <1% erythrocytes that were infected with early ring stage of *P. knowlesi*, as determined by Giemsa staining. Erythrocytes were treated with phospholipase A₂ (purified from *Naja naja* snake venom, Haefkin Institute, Bombay) essentially in the conditions described earlier⁸. After inhibiting the enzyme activity by addition of o-phenanthroline and EDTA²⁷, lipids were extracted from cells. The lipid mixture was chromatographed on silica gel G-60 TLC plates as described previously⁸ and the total phosphorus content in each spot was determined²⁸. The percentage of the total phospholipid hydrolysed after treatment of red cells with the enzyme was determined by measuring the ratio of remaining diacylglycerophospholipids to the corresponding lyso derivative. Values are expressed as mean $\pm$ s.d. <5% cells were lysed under the experimental conditions. Unsealed ghosts, prepared²⁹ from both the normal and nonparasitized cells, were also treated with the enzyme in identical conditions. Almost complete (92-98%) hydrolysis of all the diacylglycerophospholipids was observed in both the cases. n, Number of determinations.

* Three determinations on each blood sample of five uninfected monkeys and four infected monkeys (parasitaemia 10-20%).

Table 2 Labelling of erythrocyte aminophospholipids with TNBS

Sample	n*	PE (%)	PS (%)
Normal red cells	12	18.55 ± 2.87	0
Nonparasitized red cells	9	34.89 ± 2.81	16.10 ± 2.43

Labelling of erythrocytes with TNBS was carried out according to the reported procedure³⁰. The incubations were done at 20° ± 2 °C for 4, 6 and 10 h. No differences in the percentage of labelling in these experiments were observed. No detectable cell lysis occurred in all these experiments. The percentage of TNBS labelling was determined by the total phosphorus determinations and also by measuring absorbance of yellow colour at 340 nm. Values shown are mean ± s.d.. All the aminophospholipids were completely labelled when unsealed membrane ghosts from both the normal and nonparasitized cells were treated with TNBS under identical conditions. n, Number of determinations.

* Three determinations on each blood sample of four uninfected monkeys and three infected monkeys (parasitaemia 10–20%).

modified both PE (~35%) and PS (~16%) whereas in the normal cells, only PE (~19%) was labelled by the reagent. The increased amounts of PE in the outer surface of the nonparasitized cells, as compared with the normal cells, did not arise from the transmembrane movement¹⁴ of this lipid from the inner to the outer surface of the cell membrane because almost equal amounts of PE were labelled when the cells were treated with TNBS for 4, 6 and 10 h. Also, the observed differences in the amounts of labelled aminophospholipids were not due to a partial reaction with reagents, as PE and PS were completely labelled when unsealed ghosts of the two types of cells were reacted with TNBS in identical conditions. Attempts were also made to use these methods to study the nonparasitized cells obtained from blood of monkeys which developed >30% parasitaemia. Invariably, extensive haemolysis of cells occurred on

exposing them to similar concentrations of phospholipase A₂ for relatively short periods of time and also during their treatment with TNBS for >4 h. However, the amounts of PS labelled by TNBS in 4 h in such cells were significantly larger (~40%) than those observed in the nonparasitized cells of monkeys with parasitaemia (10–20%).

Accessibility of different phospholipids to phospholipases and TNBS in intact red cells has been correlated with their localization in the external leaflet of the membrane bilayer^{11,12}. From this study, it appears that parasitization of erythrocytes by the malarial parasite not only alters the phospholipid asymmetry in the parasitized cell membrane⁸, but also leads to an enormous change in the transbilayer distributions of various phospholipids in nonparasitized erythrocyte membrane. As shown in Table 1, the amounts of PC in the outer surface of the nonparasitized cells seem to be less than in normal cells. This redistribution of PC may have occurred to compensate for the increased amounts of PE and PS in the outer surface.

The above studies were carried out on a group of four infected monkeys. The same monkeys were treated with chloroquine (20 mg per kg for 3 days). This treatment completely removed circulating parasites. Similar doses of chloroquine were also given to a group of three uninfected monkeys. Determinations of transbilayer phospholipid distributions in the erythrocyte membrane on day 30 after treatment of the infected monkeys with the drug, revealed that appreciable amounts of PS (5–8%) were still present in the outer surface whereas amounts of the external PE (18–22%) in these cells were similar to those of the normal erythrocytes. Unlike the normal erythrocytes, phospholipase A₂ could still degrade PE (9–14%) in these cells.

Table 3 Concentrations of GSH in erythrocytes

Sample	n	GSH (μmol per ml packed cells)
Normal red cells	10	2.2 ± 0.7
Nonparasitized red cells*	6	1.9 ± 0.3

Concentrations of GSH in red cells were determined by the procedure of Beutler *et al.*³¹. Values are expressed as mean ± s.d.; n, number of animals.

* Parasitaemia 10–20%.

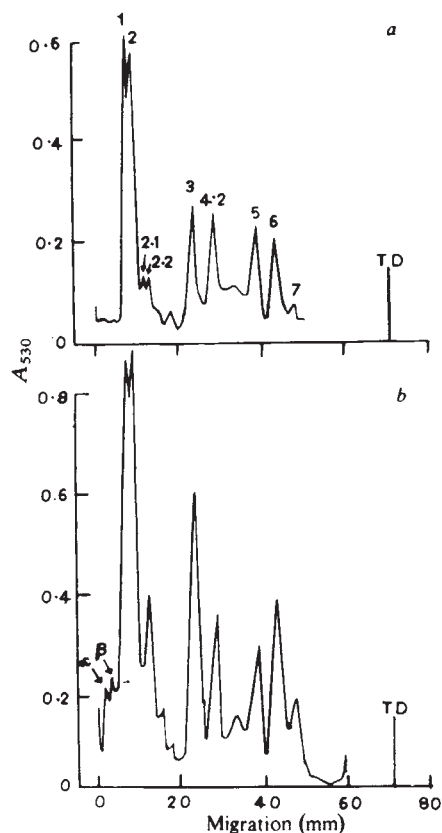


Fig. 1 Densitometer scans of gels stained for proteins with Coomassie blue after electrophoresis of red cell ghosts on polyacrylamide gels in the presence of SDS (ref. 22). *a*, Normal monkey erythrocyte ghosts; *b*, ghosts of nonparasitized erythrocytes of *P. knowlesi*-infected monkey (parasitaemia ~12%). The protein bands of the normal monkey erythrocyte are numbered according to the nomenclature of Fairbanks *et al.*²². TD, tracking dye.

However, by day 60, the amounts of PE (16–20%) and PS (none) in the external surface were normal. Chloroquine treatments of uninfected monkeys had no effect on red cell membrane phospholipid asymmetry.

Recent studies have shown that strong interactions between aminophospholipids and cytoskeletal proteins (specifically spectrin) probably determine phospholipid asymmetry in the erythrocyte membrane^{15–17}. This asymmetry in red cells is lost if the spectrin component of the cytoskeletal network becomes defective^{14,15}. Defects in the structure of spectrin may originate from decreased concentrations of glutathione (GSH) or from elevated levels of Ca²⁺ in cells^{18–21}. To look at this, membrane proteins of nonparasitized red cells were analysed by SDS-polyacrylamide gel electrophoresis²². Similar studies were also carried out on normal red cells of a group of six uninfected monkeys. The gel profiles of membrane proteins of nonparasitized erythrocytes from five different monkeys (parasitaemia 10–20%) invariably showed two additional high molecular weight protein bands (α and β , Fig. 1*b*) of variable intensity, which were absent in the gel profiles of the normal cell ghosts (Fig. 1*a*). No correlation was found between the intensity of the two bands and the extent of parasitaemia in monkeys. From a comparison between the relative intensities of protein bands present in the gel profiles of normal and nonparasitized erythrocyte ghosts, it appears that the two protein bands arise from cross-linking of spectrin. To examine whether these bands originate from oxidation of sulphhydryl groups of the protein¹⁵, the nonparasitized cell ghosts were incubated with dithiothreitol at 37 °C for 60 min before their extraction. This treatment had no effect on these bands, implying that the two high molecular weight proteins did not arise from oxidation of sulphhydryl

Table 4 Ca^{2+} contents of normal red cells of uninfected monkeys and non-parasitized erythrocytes of *P. knowlesi*-infected monkeys

Sample	n	Ca^{2+} ($\mu\text{g per ml}$ packed cells)
Normal red cells	5	0.61 ± 0.39
Nonparasitized red cells*	6	2.36 ± 1.67

Ca^{2+} content in red cells was determined by atomic absorption spectrophotometry as described by Harrison and Long³².

* Parasitaemia 10–20%.

groups of spectrin. This is further supported by our finding that almost equal concentrations of GSH were present in normal as well as nonparasitized cells (Table 3). Nevertheless, the Ca^{2+} levels in the two types of cells were significantly different (Table 4). The elevated levels of Ca^{2+} present in the nonparasitized cells are compatible with earlier findings that malaria infection in animals results in diminished levels of ATPase activity in parasitized as well as nonparasitized red cells¹⁰. A partial (or complete) deactivation of ATPase in these cells would result in the elevated levels of Ca^{2+} and consequently activation of transglutaminase which may induce cross-linking of spectrin in the nonparasitized cells^{18–20}.

Normal structure of spectrin seems essential for the invasion of red cells by the malarial parasite²³. This means that if the structure of this protein becomes defective in the total population of nonparasitized erythrocytes in a malaria-infected animal then further invasion would not proceed. That parasitaemia does regularly increase in the infected monkeys suggests that only a fraction of the total population of the nonparasitized cells becomes abnormal. This conclusion is supported by the observation that unlike the nonparasitized cells, erythrocytes containing malarial parasite at the early ring stage have PS located exclusively in the inner leaflet of the membrane bilayer⁸.

Previous findings^{9,10} indicate that the serum of malaria-infected animals contains some unknown factors which induce changes in the nonparasitized cells. We, therefore, incubated normal erythrocytes with serum of heavily infected monkeys at 37 °C for 2–3 h. Treatments of such cells with TNBS and phospholipase have so far not revealed any effect of the serum but further studies are in progress.

These observed abnormalities suggest that externalization of PS in these cells could enhance blood coagulation^{24,25} and, therefore, may cause thrombosis. Also these cells, because of their defective cytoskeletal network, would be rapidly cleared from the circulation by the spleen¹⁸, making the animals more anaemic.

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Restriction of alternative complement pathway activation by sialosylglycolipids

Noriko Okada*, Tatsuji Yasuda† & Hidechika Okada‡

* Department of Bacteriology, The Kitasato Institute, Shirokane, Tokyo 108, Japan

† Department of Cell Chemistry, Institute of Medical Science, University of Tokyo, Shirokanedai, Tokyo 108, Japan

‡ Virology Division, National Cancer Center Research Institute, Tsukiji, Tokyo 104, Japan

Although sheep erythrocytes do not activate the alternative complement pathway (ACP) of human serum, the erythrocytes gain a capacity to activate human ACP following removal of membrane sialic acid by neuraminidase treatment^{1–3}. Therefore, asialoglycoconjugates generated from sialosylglycoconjugates by neuraminidase treatment might be responsible for this type of ACP activation on cell membrane⁴. Alternatively certain sialosylglycoconjugates on the cell membrane might restrict the activation of ACP and removal of the terminal sialic acid from the glycoconjugates would abolish this restricting capacity and result in activation of the ACP on the cell membrane⁵. To determine which of the above two possible mechanisms is the case, we used a liposome model membrane in which glycolipids could be artificially inserted, and our results suggest that the latter of the two possible mechanisms is the case.

A standard liposome preparation was prepared with cholesterol and dimyristoylphosphatidylcholine (DMPC) at an equimolar ratio. To prepare liposomes with test glycolipids inserted, cholesterol, DMPC and the test glycolipid were mixed in chloroform/methanol (2:1) at a molar ratio of 1.0:1.0:0.1 and the solvent evaporated, leaving a lipid film in a rotatory evaporation flask. On the dried film of lipid, 0.2 M carboxyfluorescein (CF) was added and the lipid dispersed at 60 °C by vigorous shaking. Thus prepared liposomes were washed with gelatin veronal-buffered saline (GVB, pH 7.4) by centrifugation at 15,000 r.p.m. for 20 min. The liposomes with inserted

Table 1 Chemical structures of glycolipids used

Glycolipid	Chemical structure
GM3	NA-Gal-Glc-ceramide
GM1	Gal-GalNAc-Gal-Glc-ceramide
	NA
SPG	NA-Gal-GlcNAc-Gal-Glc-ceramide
CMH	Gal-ceramide
CDH	Gal-Glc-ceramide
CTH	Gal-Gal-Glc-ceramide
GA2	GalNAc-Gal-Glc-ceramide
GA1	Gal-GalNAc-Gal-Glc-ceramide
Globoside	GalNAc-Gal-Gal-Glc-ceramide
PG	Gal-GlcNAc-Gal-Glc-ceramide

SPG, sialosylparagloboside; CMH, galactocerebroside; CDH, lactosylceramide; CTH, ceramidetrihexoside; GA2, asialo-GM2; GA1, asialo-GM1; PG, paragloboside; NA, neuraminic acid; Gal, galactose; Glc, glucose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine.

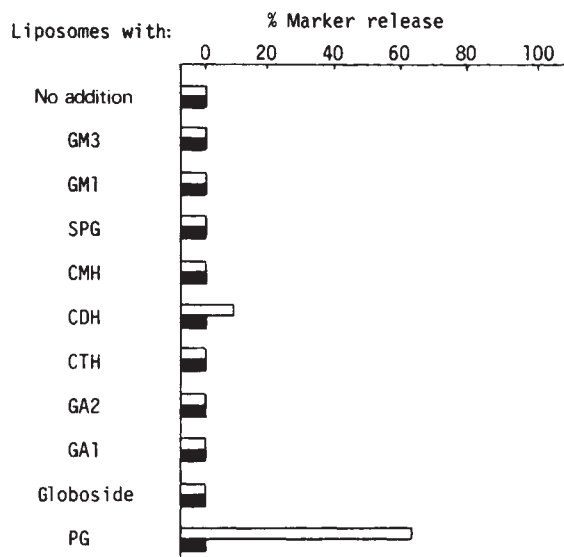


Fig. 1 Lysis of liposomes incorporated with glycolipids induced by 1:4 dilution of C4D-GPS (open bars) or human serum (solid bars) in Mg^{2+} -EGTA-GVB. To 50 μ l of a 1:4 dilution of C4D-GPS or human serum in Mg^{2+} -EGTA-GVB, 5 μ l of liposome solution (the concentration of liposome was adjusted to correspond to 0.05 mM phosphorus) in GVB were added and the mixture incubated at 25 °C for 60 min. After incubation, 2.0 ml of EDTA-VBS were added to each reaction mixture and the fluorescent intensity of CF release determined by excitation at 490 nm and emission at 520 nm. The 100% marker release was achieved by lysing liposomes with Triton X-100 as described previously⁷. Human serum did not lyse liposomes incorporated with any of the glycolipids. Although C4D-GPS lysed liposomes with paragloboside (PG), antibody against PG was detected. After heat inactivation at 56 °C, the C4D-GPS was diluted in GVB²⁺ (GVB containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$) and incubated with PG-inserted liposomes in the presence of fresh human serum to detect antibody activity to PG by classical complement pathway-mediated liposome lysis. Antibody against PG was detected up to a 1:263 dilution of the heated C4D-GPS. Therefore, no glycolipids were found which induce ACP-mediated liposome lysis by C4D-GPS or human serum in the absence of antibody reaction.

glycolipids were then incubated at 25 °C for 60 min with C4-deficient guinea pig serum (C4D-GPS) or human serum diluted 1:4 in Mg^{2+} -EGTA-GVB (GVB containing 2 mM $MgCl_2$ and 10 mM ethyleneglycol-bis(β -aminoethyl ether) N,N' -tetraacetate, pH 7.4). Mg^{2+} -EGTA-GVB was used to inhibit complement activation via the classical pathway which requires Ca^{2+} at the reaction of the first component of complement, C1. After incubation, the amount of CF marker released from the liposomes was determined by excitation at 490 nm and emission at 520 nm following dilution with EDTA-VBS (veronal-buffered saline containing 10 mM EDTA pH 7.4) as described elsewhere^{6,7}. However, no glycolipid including the asialo-type glycolipids such as CDH, GA1 and GA2 (see Table 1 for their structure) made liposomes reactive with guinea pig or human complement via the alternative pathway except where the serum used for the complement source contained natural antibody against the glycolipid inserted (Fig. 1). These results indicated that glycolipids including asialo-type glycolipids have no capacity to activate ACP in the absence of specific antibody.

Recently we found that liposomes inserted with trinitrophenylaminocaproyldipalmitoylphosphatidylethanolamine (TNP-Cap-DPPE) can be lysed by guinea pig complement via ACP activation⁷. Therefore, glycolipids were inserted onto the membrane of liposomes with TNP-Cap-DPPE (TNP-Cap-liposomes) to examine the effect of the glycolipids on the ACP-activating capacity of TNP-Cap-liposomes. TNP-Cap-liposomes with inserted test glycolipids were composed of cholesterol, DMPC, TNP-Cap-DPPE and the test glycolipids at a molar ratio of 1.0:1.0:0.05:0.1, respectively. The reactivity

of the liposomes with guinea pig ACP was examined by the release of CF marker from liposomes as described previously⁷. Sialosylglycolipids [GM3, GM1 and sialosylparagloboside (SPG)] inserted onto the liposome membrane of TNP-Cap-liposomes markedly inhibited the lysis of the liposomes by C4D-GPS in Mg^{2+} -EGTA-GVB (Fig. 2). It was possible that sialosylglycolipids such as GM3 made the liposomes resistant to complement attack and that the TNP-Cap-liposomes with the said glycolipids were not lysed by complement although they activated guinea pig ACP. However, this was not the case as the insertion of GM3 to TNP-Cap-liposomes also suppressed the capacity of the liposomes to consume guinea pig complement. Briefly, C4D-GPS diluted 1:2 in Mg^{2+} -EGTA-GVB was mixed with an equal volume of TNP-Cap-liposomes, TNP-Cap-liposomes with GM3, TNP-Cap-liposomes with CDH, and liposomes composed of only DMPC and cholesterol. Those liposomes were not carrying the CF marker and had been suspended at 5 mM phosphorus of DMPC. After incubation at 25 °C for 60 min, the reaction mixtures were centrifuged at 15,000 r.p.m. for 20 min. The supernatants were diluted in Mg^{2+} -EGTA-GVB and incubated with TNP-Cap-liposomes to determine the residual lytic activity on the liposomes via ACP activation. Preincubation of C4D-GPS with TNP-Cap-liposomes and TNP-Cap-liposomes containing CDH consumed over 90% of the complement activity while pretreatment with

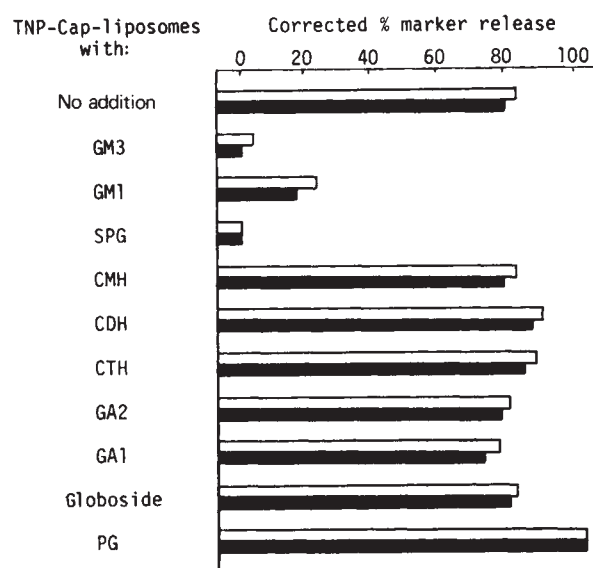


Fig. 2 Lysis of TNP-Cap-liposomes with inserted glycolipids by 1:4 (open bars) or 1:8 (solid bars) dilutions of C4D-GPS in Mg^{2+} -EGTA-GVB. Liposomes were incubated at 25 °C for 60 min with C4D-GPS and CF marker release was determined as described in Fig. 1 legend. The extent of liposome lysis was expressed as the corrected per cent marker release which was calculated as a percentage of the maximum release of CF by complement-mediated liposome lysis. The maximum release was determined by lysing liposomes with a 1:10 dilution of Hartley guinea pig serum in the presence of heat-inactivated rabbit anti-serum to TNP in GVB²⁺. The maximum release was approximately 70% of the total releasable CF in the liposomes by Triton X-100. We used the corrected per cent marker release, as the extent of possible release by complement attack varied to some degree among liposome preparations. Sialosylglycolipids (GM3, GM1 and SPG) exhibited remarkable inhibition of lysis in Mg^{2+} -EGTA-GVB by C4D-GPS in liposomes to which sialosylglycolipids were inserted. Enhanced lysis of TNP-Cap-liposomes with PG was due to the presence of antibody to PG in the C4D-GPS. TNP-Cap-liposomes also activate the classical complement pathway (CCP) of guinea pig⁷ as well as ACP. Therefore, TNP-Cap-liposomes with glycolipids were also incubated with Hartley guinea pig serum in GVB²⁺ to determine the effect of glycolipids on CCP-mediated liposome lysis. However, no inhibition of the CCP-mediated liposome lysis was noted upon insertion of the sialosylglycolipids, indicating that they inhibit complement activation via the ACP but not by the CCP.

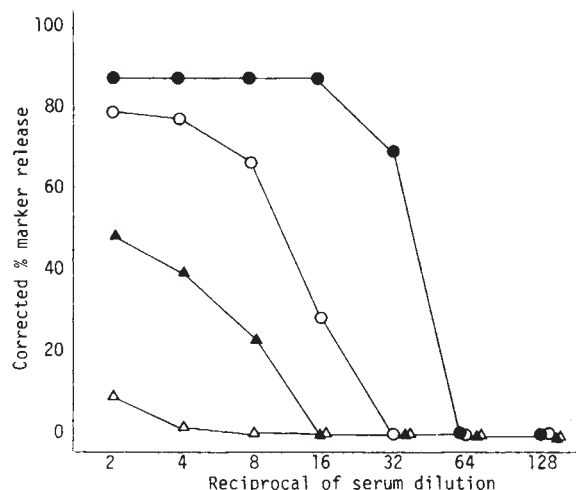


Fig. 3 Lysis by C4D-GPS of TNP-Cap-liposomes having incorporated varying amounts of GM3 onto the membrane. The membrane compositions of the liposomes were cholesterol/DMPC/TNP-Cap-DPPE/GM3 = 1.0:1.0:0.05:0.1 (Δ), 1.0:1.0:0.05:0.03 (▲), 1.0:1.0:0.05:0.01 (○), or 1.0:1.0:0.05:0 (●). Liposomes were incubated with C4D-GPS serially diluted in Mg^{2+} -EGTA-GVB and lysis was determined by CF marker release. GM3 incorporated liposome lysis was inhibited in a dose-dependent manner.

TNP-Cap-liposomes containing GM3 consumed only 40% of the complement activity. This result indicated that incorporation of GM3 onto TNP-Cap-liposomes inhibited the ACP activation on the liposomes.

On the other hand, CDH and GA1 which were asialo-type glycolipids of GM3 and GM1 respectively, did not affect the ACP-activating capacity of TNP-Cap-liposomes to which they were inserted. This suggests that sialosylglycolipids on cell membranes restrict the activation of ACP, and that removal of sialic acid from the glycolipid abolishes the restricting capacity which allows the activation of ACP on cell membrane.

To examine the amount of GM3 required for the restriction of ACP activation on TNP-Cap-liposomes, varying quantities of GM3 were inserted onto the liposomes. GM3 incorporated onto TNP-Cap-liposomes at a molar ratio to DMPC of as low as 0.01 can still inhibit ACP activation by TNP-Cap-liposomes (Fig. 3).

As sialosylglycolipids create a negative potential on the liposome surface, it was considered that the negative potential might play some part in the restriction of ACP activation on TNP-Cap-liposomes. However, positively charged amphiphiles such as stearylamine, and negatively charged ones such as dicetylphosphate and dipalmitoylphosphatidic acid, had no effect on ACP activation by the TNP-Cap-liposomes to which they were inserted⁷. Therefore, the restricted ACP activation on TNP-Cap-liposomes with sialosylglycolipids was not merely due to the negative potential on the membrane caused by the glycolipids. Sialosylglycolipids might interact with C3b molecules generated by the initial C3 convertase, C3(H₂O)Bb, which is made in serum in physiological conditions by the reaction of spontaneously hydrolysed C3 (at thiolester bond in α -chain) with factor B (ref. 8), and the C3b molecules interacted with sialosylglycolipids might be promptly inactivated by regulatory proteins in serum, factors H and I, resulting in the inhibition of the amplification reaction of C3b molecules which would otherwise form new C3 convertases with factor B, causing a positive feedback circuit in ACP activation^{9,10}.

As demonstrated above, sialosylglycolipids, and possibly sialosylglycoproteins, are thought to have a role in restricting undesirable amplification of ACP reaction, especially on self cell membranes. However, in addition to the glycolipid-mediated restriction of ACP activation on the cell membrane, some other mechanisms in the regulation of ACP activation on cell membranes such as C3b receptor-mediated restriction¹¹⁻¹³ remains

to be studied, as neuraminidase-treated cells do not activate homologous ACP^{11,14} although they activate heterologous ACP¹⁻³. This may lead to the clarification of the mechanism(s) by which complement recognition of the normal self cell membrane is achieved and how restriction of the complement reaction is maintained.

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Is nasal adenocarcinoma in the Buckinghamshire furniture industry declining?

E. D. Acheson, P. D. Winter, E. Hadfield & R. G. Macbeth

MRC Environmental Epidemiology Unit, University of Southampton, Southampton General Hospital, Southampton SO9 4XY, UK and the Department of Ear, Nose and Throat Surgery, High Wycombe General Hospital, Buckinghamshire HP11 2TT, UK

Since adenocarcinoma of the epithelium of the nasal cavity and accessory sinuses was recognized to occur more commonly in woodworkers in the British furniture industry than in other men^{1,2} and was prescribed as an industrial disease³, it has been found to occur in the furniture industry in many other countries. Here we review the incidence of the disease in Buckinghamshire up to the end of 1981 and report that skilled furniture makers such as wood machinists and cabinet and chair makers have experienced a cumulative lifetime risk of having nasal adenocarcinoma of at least 1 in 120 during the period studied. An analysis according to birth cohorts suggests that the disease may have reached its peak in men who entered the industry in the years 1915-24 but the apparent decline in incidence in men entering thereafter is not statistically significant. We argue that the carcinogen is almost certainly present in wood dust and point to the absence of recent quantitative data about dust levels in British furniture factories.

We studied the cases of nasal adenocarcinoma registered with the Oxford Regional Cancer Register between 1945 and the end of 1981. The Register includes data from the county of Buckinghamshire and the town of High Wycombe, one of the centres of the British furniture industry. Our estimates of the working population at risk have been derived from a register of 5,371 past and present workers in the Buckinghamshire furniture industry⁴ assembled (with the cooperation of the High Wycombe Furniture Manufacturers Association) by means of a letter sent to all member firms. Nine firms kept records containing sufficient identifying data to allow a follow-up. The

Table 1 SCIR for nasal adenocarcinoma in four birth cohorts of Buckinghamshire furniture workers

Birth cohort	Median year of entry to industry	Observed cases	Expected cases	SCIR (95% confidence intervals)
1890-99	1910	5	9.26	54 (18-126)
1900-09	1920	23	17.33	133 (84-199)
1910-19	1930	16	15.03	106 (61-173)
1920-29	1940	4	6.38	63 (17-161)
Total		48	48.00	100

register consists of men whose details had been recorded and retained by at least one of the nine firms and who had worked in one or more of these firms before the end of 1968. Men born after the beginning of 1940 were excluded. The nine firms comprise only 40% of the workforce in the Buckinghamshire furniture industry but are assumed to be representative of age, occupation and duration of service.

Man-years at risk were calculated for the periods 1945-68 and 1969-81, in 10-yr age groups. As the men on the register have so far only been followed up in respect of death from all causes to the end of 1968, the man-years at risk for 1969-81 were adjusted by the Buckinghamshire age-specific death rates taken from the 1969-73 Area Mortality Tables. The man-years at risk in the Buckinghamshire furniture industry for the period 1945-81 were then obtained by multiplying adjusted data from the register by the factor 2.5.

Standardized cohort incidence ratios (SCIR) were computed after Beral *et al.*⁵. Overall age-specific incidence rates were applied to the man-years at risk for individual cohorts to obtain expected numbers of cases, and confidence intervals were computed by assuming the observed number of cases to be a Poisson variable with expectation equal to the expected number of cases.

A total of 53 men who had worked at some time in the Buckinghamshire furniture industry are known to have developed nasal adenocarcinoma between the end of the Second World War and the end of 1981. Three of these (two cabinet makers and a machinist) had left Buckinghamshire before developing the tumour and were considered further only in computing the cumulative life risk of nasal adenocarcinoma in skilled furniture workers. Of the 50 men remaining, 16 were cabinet or chair makers, 22 were wood machinists including 2 turners, 5 were veneerers, 3 were sanders or benchmen and 2 were French polishers. Two were unskilled: a labourer in the sawmill of a furniture factory and a man who maintained woodworking machinery.

Although there were more cases of nasal cancer in 1960-69 (20 cases) and 1970-79 (17 cases) than in 1950-59 (8 cases), the age at which the tumours were diagnosed increased over the period. The proportions of patients over 55 yr old for the periods 1945-64 and 1965-81 were significantly different ($\chi^2 = 11.4$, 1 d.f.; $P < 0.01$).

In Table 1 the 48 skilled men have been allocated to birth cohorts. The results show that the incidence ratio for nasal adenocarcinoma increased to a maximum for the men born

during 1900-09 (whose median year of entry to the industry was ~1920) and has declined thereafter. However, the decline is not statistically significant ($\chi^2 = 2.14$, 1 d.f.; $P > 0.1$)⁶. Neither computing the expected numbers on the basis of 5-yr as opposed to 10-yr age groups, nor excluding the 1890-99 cohort from the analysis alters the conclusion that there was a decline in the incidence experienced in the last three cohorts, but that it is not statistically significant.

The incidence of nasal adenocarcinoma among skilled woodworkers in the furniture industry—SCIR 147 (95% confidence interval = 108-194)—was more than 12 times that among the other employees in the industry (SCIR 12; 95% confidence interval = 0-32). No significant difference was found between the incidence in the two principal groups of skilled woodworkers, cabinet makers and wood machinists. Those affected were all men who had worked in close proximity to cutting or sanding, which creates large quantities of wood dust. The risk of a skilled woodworker in the Buckinghamshire furniture industry suffering from a nasal adenocarcinoma in his lifetime during the period of study has thus been about 1 in 120. This value is probably an underestimate as it is unlikely to include all the men who developed the tumour after leaving the area. During 1945-81 no case of adenocarcinoma is known to have occurred in a carpenter or joiner or in a wood machinist working outside the furniture industry in Buckinghamshire, although there are at least as many such woodworkers as in the furniture industry. Only five cases of adenocarcinoma occurred in Buckinghamshire men in other occupations during the same period.

For 40 of the 48 skilled woodworkers who developed nasal adenocarcinoma, we were able to obtain information about the period of time spent in the furniture industry. Two men had worked less than 5 yr, three between 5 and 20 yr, and 35 had worked more than 20 yr in the industry (Table 2). A comparison of those who had spent less than 20 yr in the industry with those who had worked 20 yr or more, revealed SCIR of 31 (95% confidence interval = 10-72) and 147 (confidence interval = 102-204), respectively. The risk of nasal cancer is thus significantly greater in men who have served longer in the industry.

None of the cases reported in Buckinghamshire presented less than 25 yr after first exposure. In the 42 patients for whom there was sufficient information, the average period from first exposure to diagnosis was 43.9 yr. Our best estimate of the temporal pattern of nasal adenocarcinoma occurrence in the Buckinghamshire furniture industry (Table 1) suggests that the epidemic reached its peak in skilled men born during the years 1900-09 (who entered apprenticeship between 1915-25) and has begun to decline in those entering in 1924-35 and 1935-44. This pattern will have been distorted if the ascertained proportion of the total number of cases occurring altered during the period. As the Oxford Cancer Register was not fully effective until 1956, it is possible that our estimate of the incidence in the 1890-99 cohort is low. However, a search of the High Wycombe death registers suggests that the disease was extremely rare before the Second World War². In any event, exclusion of the 1890-99 cohort from the analysis does not alter the apparent decline in the incidence of nasal cancer in those entering the industry since 1925.

It is possible also that the proportion of cases brought to our attention has increased recently; such a distortion would reduce the impact of a real decline in the disease. One of us (R.G.M.) has offered a screening service to furniture workers in the High Wycombe area since 1969; as a result of this, 4 of 19 patients with nasal adenocarcinoma in furniture workers resident in Buckinghamshire have been diagnosed since 1970. It seems likely that these tumours would otherwise have been diagnosed later in the illness. The fact that, recently, nasal cancer has occurred in older men is in part explained by the aging of the workforce. Another possible source of bias is our assumption that the age distribution of the men who worked for the nine firms is representative of Buckinghamshire furniture workers as a whole.

Table 2 Cases of nasal adenocarcinoma observed and expected and SCIR in Buckinghamshire furniture workers by duration of work in the industry

Years of work in industry	Observed cases 1945-79	Expected cases	SCIR (95% confidence intervals)
Under 5	2	5.56	36 (4-130)
5-9	1	3.47	29 (1-161)
10-19	2	7.14	28 (3-101)
20+	35	23.84	147 (102-204)
Total	40	40.00	100

See text for method of computation of man-years at risk.

The fact that the furniture workers with nasal cancer whose cases are reported here had first entered the furniture industry before the Second World War does not necessarily mean that the carcinogen is no longer present in the working environment. Three cases of nasal adenocarcinoma in men who entered the furniture industry in other areas of England shortly after the Second World War have been reported to us and cases in men who entered the industry during the war have been reported in France and Italy^{7,8}.

Nasal adenocarcinoma is now known to have been a special risk to furniture makers elsewhere for several decades and in Britain since 1920 and possibly earlier. This widespread occurrence, together with the fact that woodworking machinists (who saw the timber) and the cabinet and chair makers (who shape, finish, sand and assemble the furniture) experience similar risks make it unlikely that the tumours are due to a chemical agent applied to the wood at a particular stage of the process but more probably to a substance in wood dust itself. Occupational histories show that men exposed exclusively to beech and oak have developed tumours, but a wider range of hardwoods is probably implicated. It is unknown whether the absence of an excess risk of tumours in carpenters and joiners is due to the exposure of these men to low concentrations of dust particles of the appropriate size or because the carcinogen is absent from soft woods.

Prolonged inhalation of dust by furniture workers has been shown to impair mucociliary clearance and to lead to squamous metaplasia. Deposits of dust have been observed in the noses of furniture workers, on the mucosa close to the ethmoid bulla where the tumours are believed to originate⁹. The nature of the substances in wood dust which bring about these changes and which initiate carcinogenesis is unknown. Far from being an inert material as was believed previously, wood contains a wide range of biologically active substances including flavonols, other phenolic compounds and many terpenoids, some of which probably exert a protective function in timber as natural insecticides and fungicides. It is possible that one or more of these may be carcinogenic. Another possibility is that carcinogens may be formed by pyrolysis during machining. As nasal cancer in boot and shoe operatives is limited to men and women exposed to dust associated with preparing and finishing leather for heels and soles—which is usually tanned with vegetable extracts including extracts of wood—it is possible that these cancers also may be caused by a carcinogen derived from wood¹⁰. Systematic studies of the carcinogenicity of wood and leather dust are needed.

Although nasal cancer in furniture makers was prescribed as an industrial disease in 1969, there are no data indicating whether dust levels in British furniture factories have subsequently declined. A study of Buckinghamshire furniture factories published in 1974 showed that the average dust concentration was $\geq 5 \text{ mg ml}^{-1}$ (ref. 11). In 1976 a regulation was introduced in the United Kingdom requiring 'efficient exhaust ventilation' in furniture factories¹² but it is unknown whether this regulation has brought an improvement in dust levels.

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Loss of intervening sequences in genomic mouse α -globin DNA inserted in an infectious retrovirus vector

Kunitada Shimotohno* & Howard M. Temin

McArdle Laboratory, University of Wisconsin, Madison, Wisconsin 53706, USA

Genes lacking the intervening sequences that are present in otherwise apparently homologous genes have been found in some highly oncogenic retroviruses (some viral oncogenes) and in the normal cell genome (cDNA genes, pseudogenes or processed genes)^{1–10}. Such cDNA genes might have arisen by reverse transcription of the spliced (intervening sequences removed) RNA transcript of the homologous genes. Another possible way for genes with intervening sequences to lose such sequences would be during replication in a retrovirus vector. To test this hypothesis we inserted genomic mouse α -globin DNA containing two intervening sequences¹¹ into the DNA of an infectious retrovirus vector¹² and report here that the intervening sequences were removed from DNA of progeny virus during virus replication.

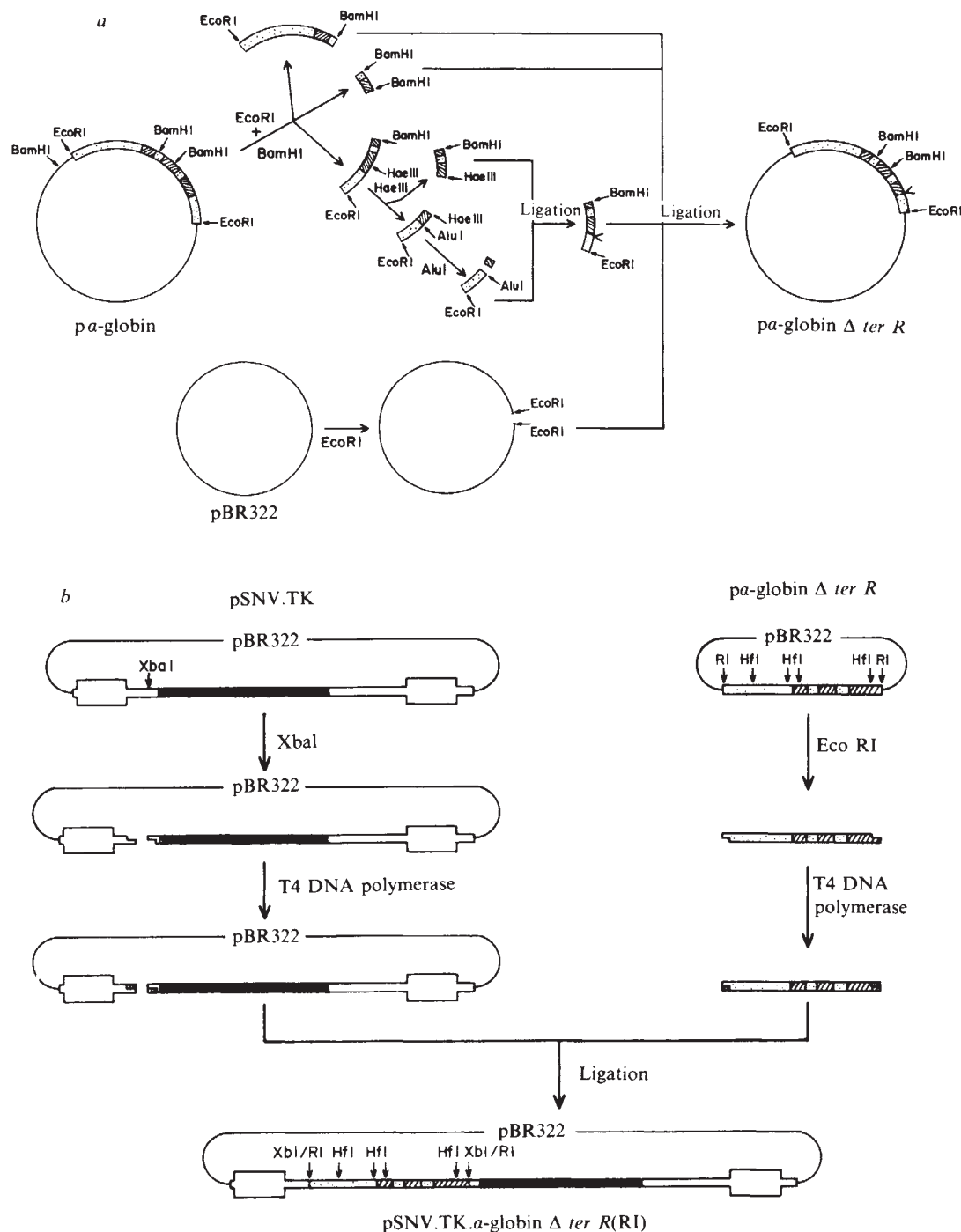
We previously found that removal of the 3' terminus of a thymidine kinase (TK) gene before its insertion in our infectious spleen necrosis virus (SNV) vector increased the recovery of infectious virus¹². Therefore, we prepared DNA of mouse α -globin without 3'-terminal sequences including the site of poly(A) addition (α -globin Δ terR gene) by removing 130 base pairs (bp) from the gene. The sequences removed were from 25 bp after the end of the coding sequences for protein (*Hae*III cleavage site) to 65 bp after the end of the sequences for mRNA (*Alu*I cleavage site) (Fig. 1a, also see map in Fig. 3).

The *Eco*RI- or *Hin*FI-digested α -globin Δ terR was then inserted into the *Xba*I cleavage site 40 bp (13 amino acids) inside the *gag* gene¹³ of a herpes simplex virus thymidine kinase Δ terR gene-containing retrovirus vector in which retrovirus sequences from approximately 1 to 7 kbp had been deleted¹⁴. (This vector is produced in mixed infection at approximately the same rate as a non-defective helper retrovirus¹⁴.) The strategy used for this insertion maintained both the *Xba*I and the *Eco*RI cleavage sites, pSNV-TK- α -globin Δ terR(RI), or *Hin*FI cleavage sites, pSNV-TK- α -globin Δ terR (*Hin*FI), at the ends of the vector and the α -globin Δ terR DNA (map in Fig. 1b).

To recover infectious virus carrying the α -globin DNA, pSNV-TK- α -globin Δ terR(RI) DNA was co-transfected into chicken cells with one tenth as much DNA of a provirus clone of a non-defective helper virus, reticuloendotheliosis virus strain A (REV-A). The virus produced was first assayed by its thymidine kinase transforming activity in buffalo rat liver (BRL) TK⁻ or chicken TK⁻ cells¹². SNV-TK- α -globin Δ terR(RI) was recovered at ~10% the yield of the parental SNV-TK; and SNV-TK α -globin Δ terR (*Hin*FI) was produced at about 30% of the yield of SNV-TK (data not shown). Therefore, sufficient recombinant thymidine kinase-containing virus was produced for a direct examination of its structure. Virus recovered 5 days after transfection with pSNV-TK- α -globin Δ terR(RI) $\sim 10^4$ – 10^5 TK-forming units per ml) was used to infect fresh chicken embryo fibroblasts. Three days later unintegrated linear viral DNA was prepared, digested with *Hin*FI, *Xba*I or *Eco*RI, and analysed (Fig. 2). Two bands, of 1.9 and 1.7 kbp, that hybridized to α -globin DNA were found after digestion with *Xba*I or *Eco*RI. The upper band is the same size as the parental α -globin Δ terR; the lower band about 250 bp smaller (see maps in Fig. 3). This difference in size is consistent with the absence of both intervening sequences from the DNA in the lower band (IVS-1 is 122 bp and IVS-2 is 134 bp¹¹). Digestion with *Hin*FI also resulted in two bands, of 810 and 560 bp, that hybridized

* Present address: National Institute of Genetics, Mishima, 411 Japan.

Fig. 1 Construction of recombinant DNAs. *a*, Construction of α -globin: $\Delta terR$: α -globin (200 μ g) was digested with *Eco*RI (New England Biolabs) at 37 °C for 10 h. The digest was analysed by electrophoresis into a 1% agarose gel at 30 V for 12 h. A fragment of 2 kbp (α -globin) was isolated from the gel²¹, digested with *Bam*HI (New England Biolabs), and the digest analysed by electrophoresis into a 1% agarose gel. DNA fragments of 1.2 (*Eco*RI/*Bam*HI), 0.3 (*Bam*HI/*Bam*HI), and 0.6 (*Bam*HI/*Eco*RI) kbp were isolated from the gel. The 0.6-kbp (*Bam*HI/*Eco*RI) fragment was digested then with *Hae*III (New England Biolabs), which digests between the C-terminal end of the protein and AATAAA in the untranslated region of the sequence (see map in Fig. 3), and the digest was analysed by electrophoresis into a 6% acrylamide gel. DNA fragments of 0.3 (*Bam*HI/*Hae*III) and 0.32 (*Hae*III/*Eco*RI) kbp were isolated from the gel. The fragment of 0.32 kbp (*Hae*III/*Eco*RI) was digested with *Alu*I (New England Biolabs), which digests after the site of poly(A) addition (ref. 11, also see map in Fig. 3), and the digested DNA was analysed by electrophoresis into a 6% acrylamide gel. A DNA fragment of 0.2 kbp (*Alu*I/*Eco*RI) was isolated from the gel. Equimolar amounts of the *Bam*HI-*Hae*III fragment of 0.3 kbp and the *Alu*I-*Eco*RI fragment of 0.2 kbp were ligated with T4 DNA ligase (NEN) at 24 °C for 17 h to allow ligation between the *Alu*I and *Hae*III sites. The ligated DNA (concatamers) was digested with *Bam*HI and *Eco*RI to produce a 0.5-kbp fragment having *Bam*HI and *Eco*RI sites at its ends. Equimolar amounts of a 4.3-kbp *Eco*RI-digested pBR322, which had been treated with alkaline phosphatase (BRL), the 1.2-kbp *Eco*RI-*Bam*HI fragment, the 0.3-kbp *Bam*HI-*Bam*HI fragment and the 0.5-kbp *Bam*HI-*Eco*RI fragment were ligated together. The ligated DNA was introduced into *Escherichia coli* HB101 cells and transformants carrying plasmids were grown in the presence of 25 μ g ml⁻¹ of ampicillin. Colonies which hybridized to mouse α -globin DNA were isolated and grown in L-broth to prepare plasmid DNA. The DNAs were characterized by mapping with restriction endonucleases *Pst*I, *Bam*HI and *Eco*RI (data not shown). Symbols: line, pBR 322; cross-hatched bar, α -globin mRNA coding sequences; stippled bar, noncoding sequences; V indicates deletion of 128 bp from the 3' terminus of the mouse α -globin DNA. *b*, Construction of pSNV-TK- α -globin $\Delta terR$ (RI): α -globin $\Delta terR$ DNA (100 μ g) was digested with *Eco*RI. A 2-kbp (*Eco*RI/*Eco*RI) α -globin-containing fragment from the digest was isolated after electrophoresis in a 1% agarose gel. pSNV-TK DNA (20 μ g) was digested with *Xba*I (New England Biolabs). The ends of these DNAs were filled in with T4 DNA polymerase (Bethesda Research Laboratories). The reaction mixture consisted of 330 μ M of dATP, dCTP, dGTP and dTTP, 167 μ g ml⁻¹ of bovine serum albumin, 16.6 mM of ammonium sulphate, 1 mM dithiothreitol, 6 mM MgCl₂, 50 mM Tris-HCl (pH 8.5), 5 μ g DNA fragment and 2 U of T4 DNA polymerase in 40 μ l. The reaction was carried out for 1 h at 37 °C. The reaction mixture was passed through a Sephadex G-50 column (0.7 $\times$ 12 cm) to separate unreacted materials from DNA and the DNA was then precipitated with ethanol. Equimolar amounts of these DNA fragments were ligated together. The ligated DNA was introduced into *E. coli* HB101 cells and the transformants were grown in L-broth in the presence of 50 μ g ml⁻¹ ampicillin. Plasmids carrying α -globin $\Delta terR$ DNA were isolated from the transformants and the structures of the plasmid DNAs were characterized by mapping with restriction endonucleases (data not shown). Clones prepared in this manner with T4 DNA polymerase retain the cleavage sites of both *Xba*I and *Eco*RI. Construction of pSNV-TK- α -globin $\Delta terR$ (*Hinf*I): α -globin $\Delta terR$ DNA was digested with *Hinf*I (New England Biolabs), which cuts 20 bp 3' to the sequences for the 5' end of globin mRNA, 118 bp 3' to the sequences for the carboxy-end of α -globin, and at two other sites in the 5'-noncoding region (see map in Fig. 3)¹¹. A DNA fragment of ~800 bp (*Hinf*I/*Hinf*I) containing α -globin sequences was isolated after electrophoresis in a 1% agarose gel. The ends of the *Hinf*I fragment were filled in by T4 DNA polymerase as described above. The filled-in DNA fragment and pSNV-TK that was digested with *Xba*I and had its ends filled in with T4 DNA polymerase were ligated together with T4 DNA ligase. Transformed *E. coli* HB101 cells carrying the plasmid were isolated and the plasmid DNA was mapped by restriction endonuclease digestion (data not shown). Cleavage sites of both *Xba*I and *Hinf*I were retained by this method of construction. Symbols: open bar, SNV; filled bar, herpes simplex thymidine kinase gene; other symbols as in Fig. 1a. (The sequences 3' of exon 3 are now shown cross-hatched to indicate the removal of the sequences for the 3' end of globin mRNA. The pBR322 sequences are not drawn to scale.)



to α -globin DNA (other hybridizing bands ran off the gel). The upper band contains the parental α -globin $\Delta terR$ coding sequences (A); the lower band contains 5' non-translated DNA sequences (B) and the progeny (smaller) α -globin $\Delta terR$ sequences (C). The increase in relative amount of the lower band locates the decreased size of the α -globin $\Delta terR$ DNA to the coding sequences.

Similar results were found after co-transfection with pSNV-TK- α -globin $\Delta terR$ (HinfI) and REV-A DNAs (data not shown).

To confirm the loss of intervening sequences in the progeny virus, molecular clones were made of the insert recovered from progeny virus. Closed circular viral DNA was prepared 3 days after infection of chicken cells with virus that was recovered 5 days after transfection with pSNV-TK- α -globin $\Delta terR$ (RI). This closed circular DNA was digested with *Xba*I and the α -globin $\Delta terR$ insert was cloned in the modified λ vector Charon 4A as described in Fig. 3 legend. Globin DNA-containing phage were then isolated and the inserts characterized by digestion with *Hinf*I and *Bam*HI. The results of restriction enzyme mapping are consistent with loss of the two intervening sequences in the α -globin DNA (Fig. 3). DNA sequencing then directly demonstrated that the two intervening sequences were precisely removed (data not shown).

To determine the rate of loss of intervening sequences in progeny virus, virus was collected 4–9 days after transfection and used to infect fresh chicken cells. Unintegrated linear viral DNA was then prepared 3 days after infection (7–12 days after transfection). Multiple cycles of infection had occurred in this time.

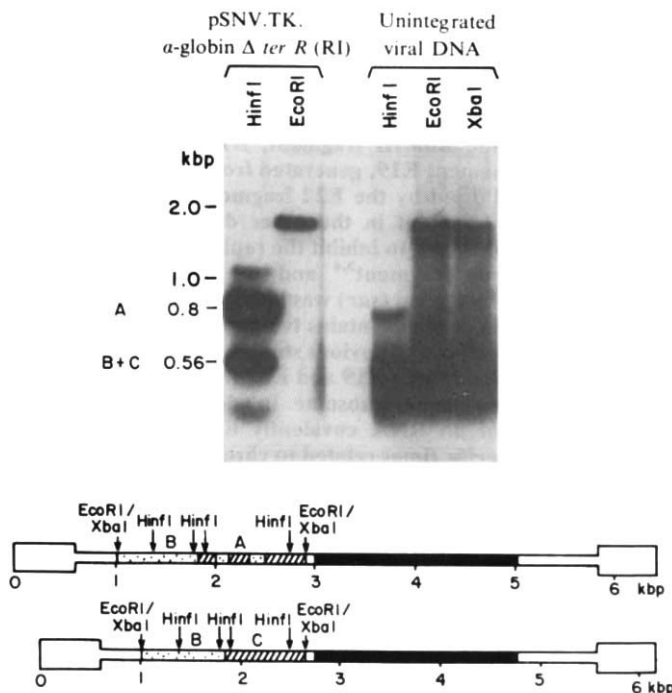


Fig. 2 Recovery of unintegrated viral DNA from chicken cells infected with recombinant virus. pSNV-TK- α -globin $\Delta terR$ (RI) DNA (10 μ g) and REV-A DNA (1 μ g) as helper were introduced into chicken fibroblastic cells in 100-mm Petri dishes in the presence of calcium phosphate. After 5 days, virus was collected from the medium and fresh chicken cells (30–100-mm Petri dishes) were infected with undiluted virus. Three days after infection, the cells were collected and unintegrated viral DNA was isolated by the Hirt procedure^{22,23}. The unintegrated viral DNA was digested with *Hinf*I, *Xba*I or *Eco*RI and analysed by electrophoresis in a 1.3% agarose gel. Each lane contained DNA from four Petri dishes. The gel was blotted onto nitrocellulose filter paper and the filter was hybridized with nick-translated ³²P-labelled globin-containing *Eco*RI fragment from α -globin $\Delta terR$ (RI). pSNV-TK- α -globin $\Delta terR$ (RI) DNA was used as a control (left two lanes). The maps of unintegrated viral DNA at the bottom represent the parental virus and progeny virus without globin intervening sequences. The smearing seen in the unintegrated viral DNA lanes is probably a result of degradation in cells killed by the virus infection²⁴. A distinct 0.56-kbp band is visible in the *Hinf*I lane of the original.

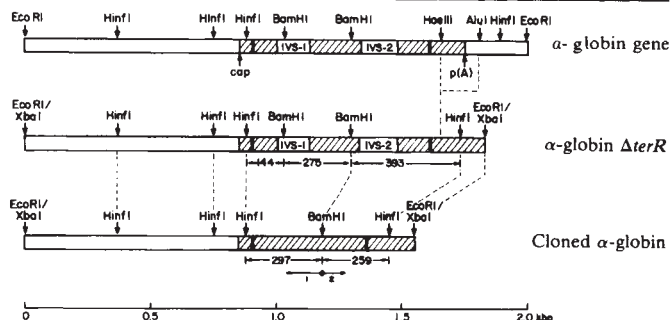


Fig. 3 Cloning and characterization of an α -globin DNA fragment from SNV-TK- α -globin $\Delta terR$ (RI). Isolation of circular unintegrated viral DNA from chicken cells infected with virus produced by chicken cells transfected with SNV-TK- α -globin $\Delta terR$ (RI) and REV-A DNAs: Virus-infected chicken cells (60–100-mm Petri dishes) were lysed with SDS 3 days after infection. Unintegrated viral DNA was isolated by the Hirt procedure. The Hirt supernatant fraction was treated with proteinase K (20 μ g ml⁻¹; Beckman) for 2 h at 37 °C and then with phenol and chloroform. One gramme of CsCl was added per ml of the preparation, and ethidium bromide was added to a final concentration of 0.1 mg ml⁻¹. The solution was centrifuged for 48 h in the Beckman 50 Ti rotor at 35,000 r.p.m. and 40 fractions were collected from the bottom of the centrifuge tube. 5 μ l of each fraction were spotted on to nitrocellulose filter paper, the DNA was denatured with 0.1 M NaOH and the filter was neutralized, heated at 80 °C for 2 h, and hybridized with ³²P- α -globin DNA. Fractions containing circular unintegrated viral DNA were collected and ethidium bromide and CsCl were removed by extraction with isopropanol and dialysis, respectively. The viral DNA was then digested with *Xba*I. Charon 4A DNA²⁵ was digested first with *Xba*I and then with alkaline phosphatase to remove terminal phosphate residues. Four molar equivalents of the insert DNA were added and the DNAs were ligated together. The ligated DNA was packaged, and the phage were used to infect *E. coli* DP50supF on NZYDT agar plates²⁵. The efficiency of isolation of positive plaques was about 10⁻³. The α -globin-containing phage were purified by serial dilution of the plaques containing the phage. DNA was extracted from purified phage, digested with *Xba*I and electrophoresed into a 1% agarose gel. The cleavage map of one of the clones is shown compared with the α -globin $\Delta terR$ DNA and the original α -globin gene¹¹. The thick vertical lines in exons 1 and 3 represent the ends of protein coding sequences. The numbers beneath the maps represent the sizes of the fragments found. Strategy used for sequencing the *Xba*I-digested globin DNA clone: the *Xba*I-digested DNA clone was digested with *Bam*HI followed by digestion with alkaline phosphatase. The DNA was labelled at its 5' ends with ³²P by T4 polynucleotide kinase (P-L Biochemicals) and then digested with *Hinf*I. DNA fragments of 300 and 260 bp were isolated and sequenced by the method of Maxam and Gilbert²⁶. The regions sequenced are indicated by arrows under the map of the cloned α -globin DNA and agree with the published sequence¹¹. IVS-1 is intervening sequence 1; IVS-2 is intervening sequence 2; 1 and 2 are the sequences of the regions corresponding to IVS-1 and IVS-2 after recovery from virus; cap is the 5' end of mouse α -globin mRNA; p(A) is the poly(A) addition site for mouse α -globin mRNA.

Figure 4 shows that virus with inserts containing intervening sequences was still present 7 days after transfection. Thus, the probability of losing the intervening sequences per replication cycle is less than 100%. However, by 9 days after transfection essentially all the viral DNA lacked the globin intervening sequences.

In a parallel experiment, virus was collected 4 days after transfection and passaged at 3-day intervals in fresh chicken cells. Before each passage, the unintegrated viral DNA was analysed as in the experiments described in Figs 2 and 4. Virus containing unspliced inserts was still detected after the first passage (7 days after the initial transfection) (data not shown). By the second passage all virus recovered contained only spliced globin DNA.

We believe that the loss of intervening sequences from these retroviral vectors occurred from genomic RNA for the following reason. In one of our constructions, pSNV-TK- α -globin $\Delta terR$ (HinfI), the *Hinf*I cleavage is ~20 bp 3' to the 5' end of sequences for globin mRNA¹¹ and so removes any promoter for globin mRNA synthesis. Progeny virus containing α -globin DNA without intervening sequences was recovered after transfection with pSNV-TK- α -globin $\Delta terR$ (HinfI) DNA (data not shown). Since no subgenomic RNA could be made, this finding indicates that splicing took place in genomic RNA rather than in globin mRNA followed by recombination with genomic RNA.

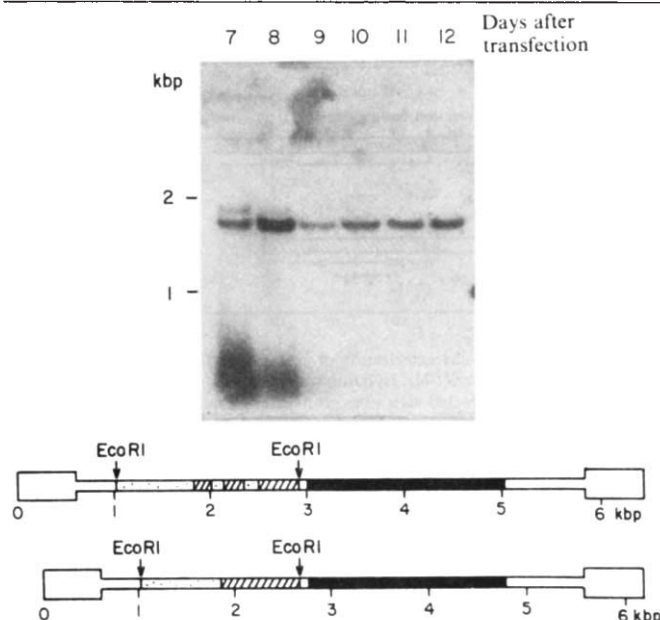


Fig. 4 Kinetics of loss of intervening sequences in recombinant virus. Virus produced by chicken cells transfected with pSNV-TK- α -globin Δ terR(R1) and REV-A DNAs was collected 4, 5, 6, 7, 8 and 9 days after transfection. Fresh chicken cells were infected with these viruses. Three days after infection (days 7–12 after transfection), unintegrated viral DNA was extracted by the procedure of Hirt^{22,23}. The DNAs were digested with *Eco*RI and analysed by electrophoresis in a 1.3% agarose gel. The gel was blotted onto a nitrocellulose filter and the filter was hybridized with nick-translated ³²P- α -globin DNA. Parallel preparations of DNA from uninfected chicken cells did not hybridize to mouse α -globin DNA (data not shown).

The difference between the normal 100% splicing of globin gene transcripts¹⁵ and the less efficient (~10%) splicing of the same sequences seen here may reflect the requirements of the retrovirus life cycle. Retroviruses normally synthesize some subgenomic mRNAs¹⁶. If all viral RNA were spliced, only subgenomic RNA and no progeny genomic RNA would be made. The retrovirus may therefore control the rate of splicing, perhaps through their 5' sequences which also control numerous other processes including encapsidation^{14,17,18}. Alternatively, the difference in cells or secondary structure of the RNA could be responsible for the difference in efficiency of splicing.

No virus containing DNA with one intervening sequence spliced out and the other remaining was found. Because the rate of loss of intervening sequences is less than 100%, this result indicates that loss of the two intervening sequences is not independent. Normal splicing of mouse β -globin mRNA precursor involves a partially spliced intermediate¹⁹.

The results reported here clearly demonstrate information transfer from DNA through spliced RNAs to DNA. They thus support the hypothesis that cDNA genes arose by reverse transcription of spliced RNA^{6-10,20}. However, it would not be correct to conclude that the results reported here indicate that retrovirus transfer is necessary for the formation of cDNA genes although it may well account for the origin of some viral oncogenes. In any case, our data illustrate how retroviruses can be used to delete intervening sequences from genomic DNAs, a process that would allow the expression of eukaryotic genes in prokaryotes.

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Ribosomal RNA genes in the replication origin region of *Bacillus subtilis* chromosome

G. Henckes*, F. Vannier*, M. Seiki†, N. Ogasawara‡, H. Yoshikawa‡ & S. J. Seror-Laurent*§

* Institut de Microbiologie, Bâtiment 409, Université Paris XI-91405, Orsay Cedex 05, France

† Department of Viral Oncology, Cancer Institute, Toshimaku, Tokyo, Japan

‡ Cancer Research Institute, Kanazawa University, Kanazawa, Japan

In *Bacillus subtilis*, DNA replication proceeds bidirectionally, commencing in the *Bam*HI fragment, B7 (see Fig. 4). The middle *Eco*RI fragment E19, generated from B7, is the first to be replicated, followed by the E22 fragment in one direction and by the E6' fragment in the other direction^{1,2}. The B7 fragment was also shown to inhibit the replication of a plasmid which carried this fragment^{3,4} and the minimal essential sequence for the inhibition (*sar*) was located to a 200-base pair (bp) region of E19, which contains two promoters, as deduced by the sequence analysis⁵. Previous studies showed that the E6' fragment was unique while E19 and E22 fragments hybridized to multiple sites on the chromosome. In addition, in the course of the analysis of an RNA covalently linked to DNA and synthesized at specific times related to chromosome initiation⁶, we found that this RNA was complementary both to the E19 and E22 fragments and to ribosomal RNA genes⁷. We show here that a ribosomal RNA gene set (*rrnO*) overlaps the first replicating fragments in one direction. The promoters for these genes are in fact the promoters of the *sar* sequence.

B. subtilis chromosomal DNA was digested to completion by *Bam*HI and *Sma*I restriction endonucleases, separated electrophoretically, and the resulting fragments were assayed by hybridization with ³²P-labelled E19 and E22. The patterns presented in Fig. 1 show that the E19 and E22 fragments hybridized with at least 7 different fragments on the chromosome, in agreement with earlier results⁴. A similar hybridization experiment was performed with the total chromosomal DNA using 16S and 23S RNA probes. The results presented in Fig. 2 showed that the E19 and E22 fragments hybridized with restriction fragments which are specific for the 16S RNA or for both 16S and 23S RNA. The bands corresponding to 14, 11 and 3.5 kbp *Bam*HI fragments, and to 7.2 and 5.4 kbp *Sma*I fragments, which are specific for the 23S RNA⁸, were absent in both E19 and E22 hybridization patterns.

To determine the precise homology between the E19 and E22 fragments and the ribosomal RNA genes, we took advan-

§ To whom correspondence should be addressed.

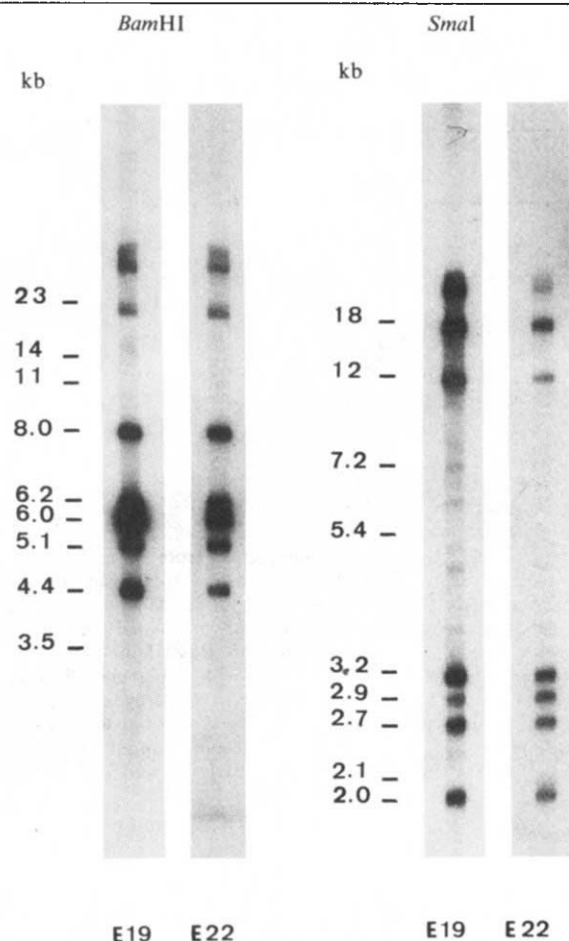


Fig. 1 Southern hybridization of E19 and E22 fragments with the *Bam*HI and *Sma*I *B. subtilis* DNA digests. Chromosomal DNA from *B. subtilis* 168 (*thy*, *trp*) was digested to completion either with *Bam*HI or with *Sma*I (New England Biolabs), electrophoresed in 0.7% agarose, transferred and fixed on nitrocellulose sheets (Millipore) according to the method of Southern¹⁹. DNA from plasmid pKY2700 containing E19 or E22 fragments⁴ was purified by caesium chloride-ethidium bromide equilibrium centrifugation followed by sedimentation on sucrose gradients and finally digested with *Eco*RI and electrophoresed in 1% agarose. After extraction from the gel, the E19 and E22 fragments were nick translated with [α -³²P]dCTP (400 Ci mmol⁻¹, Amersham France). Before mixing with ³²P-DNA the Southern transfers were incubated for 1 h at 65 °C in 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin, 100 μ g ml⁻¹ yeast tRNA 4 $\times$ SSC. Then the ³²P-labelled E19 or E22 DNA was added and, after 18 h at 65 °C, the sheets were washed in 2 $\times$ SSC, 0.5% SDS for 2 h at 65 °C, then for 1 h at 25 °C and finally in 2 $\times$ SSC for 1 h at 25 °C. After washing the sheets were exposed to X-ray film.

tage of the restriction map of a ribosomal RNA gene set recently obtained by Bott *et al.*⁹. DNA from plasmid p14B1 containing the DNA sequence coding for one of the ribosomal RNA gene clusters, was completely digested as detailed in Fig. 3. The resulting fragments were separated electrophoretically and assayed by hybridization with ³²P-labelled E19 and E22 fragments. The E19 fragment showed homology with the fragment limited by the *Hind*III and *Eco*RI sites and containing the coding sequence for the 5'-terminus of 16S RNA (Fig. 3). The E22 fragment showed homology with the fragment limited by *Eco*RI sites and containing the coding sequence for the 3'-terminus of 16S RNA.

The restriction maps independently constructed by Bott *et al.*⁹ and Seiki *et al.*¹, for one of the ribosomal RNA gene clusters and the replication origin region respectively are remarkably similar, as shown in Fig. 4. Most of the differences could be explained by a small displacement of several of the sites. In fact, some heterogeneity has been observed in the organization of the different ribosomal RNA genes (ref. 10 and G. C. Stewart,

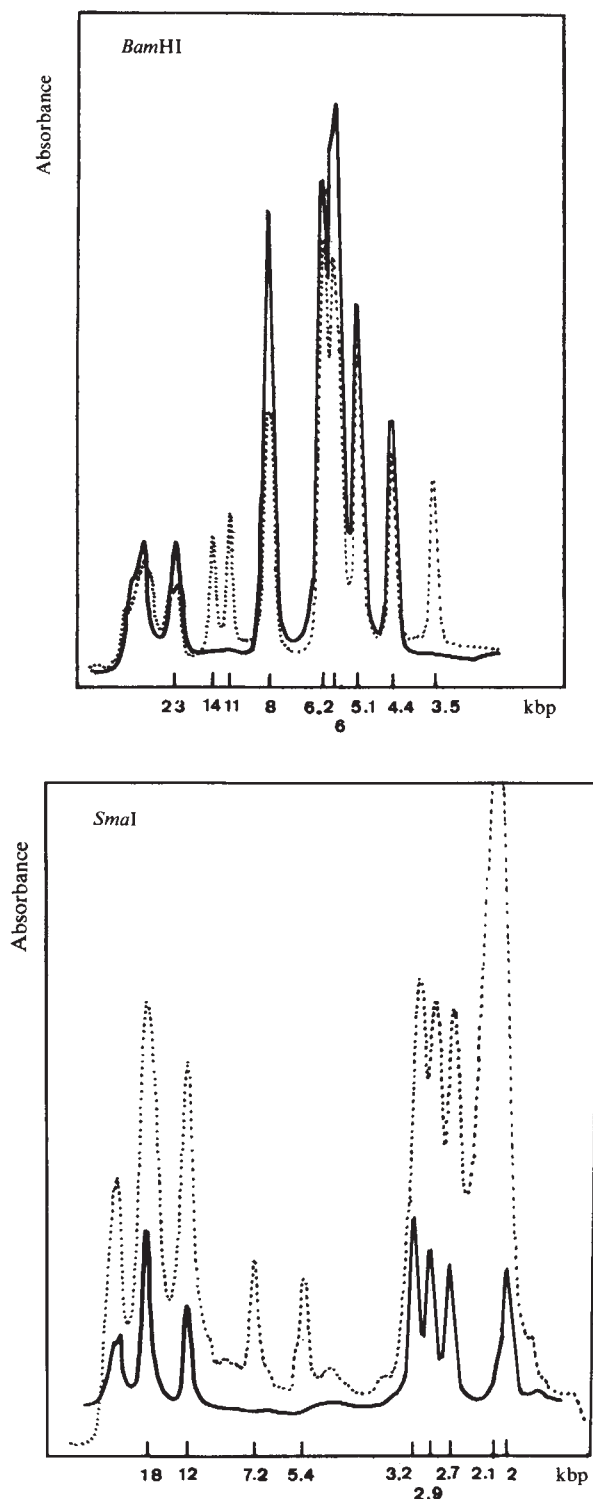


Fig. 2 Comparison of hybridization patterns obtained with ³²P-E19 and E22 fragments and ³²P-ribosomal RNA (16S + 23S). E19 and E22 fragments were labelled with ³²P and hybridized with the *Bam*HI and *Sma*I *B. subtilis* DNA digests as described in Fig. 1 legend. The 16S and 23S ribosomal RNAs were purified from a nucleic acid extract by sedimentation through 15–30% sucrose gradients and labelled at their 5' termini with [α -³²P]ATP (3,000 Ci mmol⁻¹, Amersham France) and T4 polynucleotide kinase. The ³²P-labelled 16S and 23S were incubated with the Southern transfers for 18 h at 68 °C in 3 $\times$ SSC and 0.1% SDS. After hybridization the sheets were washed in 3 $\times$ SSC for 1 h at 37 °C, then in 3 $\times$ SSC for 4 h at 48 °C. The sheets were exposed to X-ray film and the absorbance of the film image was measured using a densitometer (Kipp et Zonen). ³²P-E19 or ³²P-E22 fragments (—); ³²P-ribosomal RNA (16S + 23S) (·····).

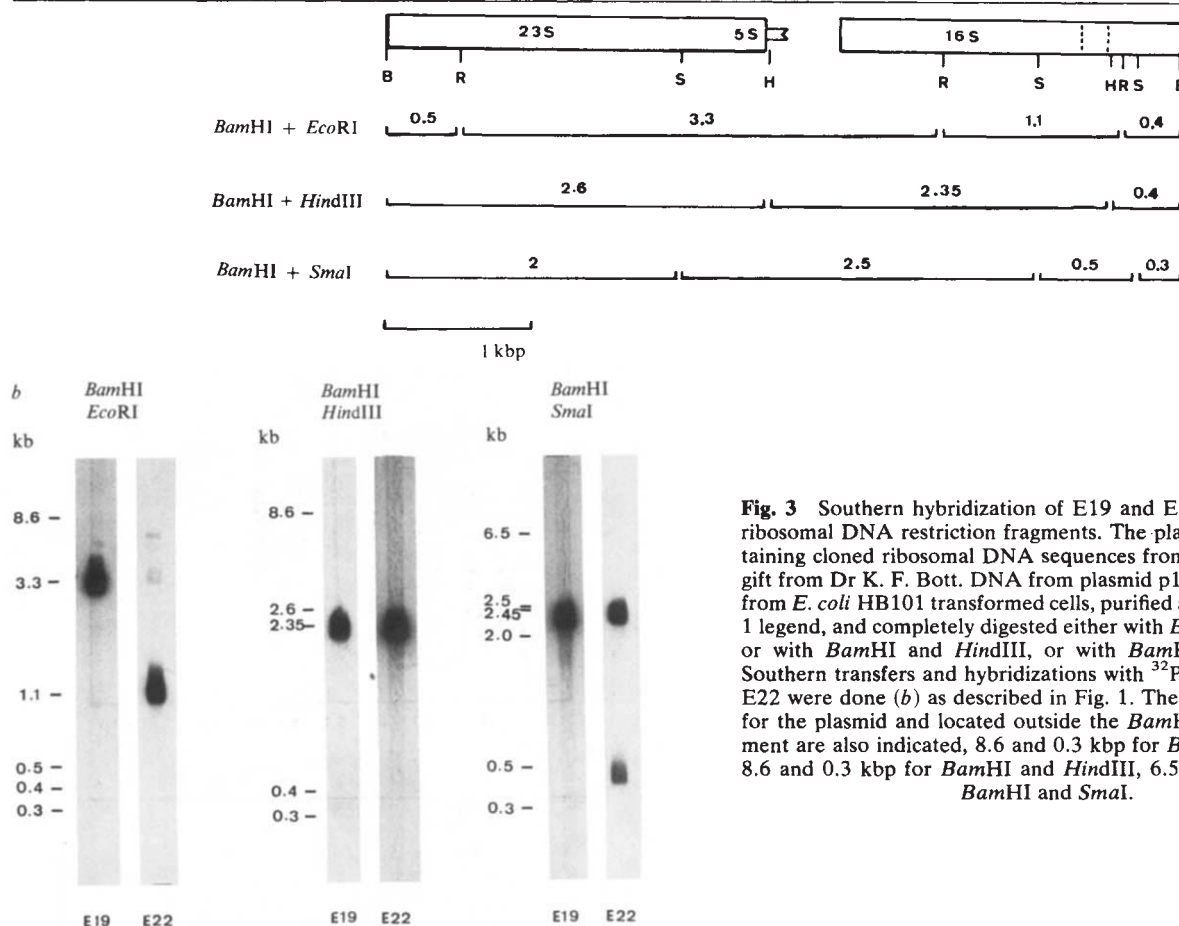


Fig. 3 Southern hybridization of E19 and E22 fragments with ribosomal DNA restriction fragments. The plasmid p14B1, containing cloned ribosomal DNA sequences from *B. subtilis*, was a gift from Dr K. F. Bott. DNA from plasmid p14B1 was prepared from *E. coli* HB101 transformed cells, purified as described in Fig. 1 legend, and completely digested either with *Bam*HI and *Eco*RI, or with *Bam*HI and *Hind*III, or with *Bam*HI and *Sma*I (a). Southern transfers and hybridizations with 32 P-labelled E19 and E22 were done (b) as described in Fig. 1. The fragments specific for the plasmid and located outside the *Bam*HI-generated fragment are also indicated, 8.6 and 0.3 kbp for *Bam*HI and *Eco*RI, 8.6 and 0.3 kbp for *Bam*HI and *Hind*III, 6.5 and 2.45 kbp for *Bam*HI and *Sma*I.

personal communication). Moreover, the similarity observed concerns not only the region covering the E19 and E22 fragments but also the region beyond the E22 fragment, suggesting the presence of a complete ribosomal RNA gene set in the region of the chromosome origin.

From the data obtained in this study, it seems likely that a 500-bp region, which has been shown to be responsible for the inhibitory effect on plasmid replication⁴, could include the ribosomal RNA promoters and a sequence of nucleotides which is normally removed from the precursor 16S RNA to form the mature molecule¹¹. Recently, the nucleotide sequence of this region has been determined and has revealed the presence of two tandem promoters located 98 bp apart⁵. The sequence of the analogous region of the ribosomal RNA genes cloned in p14B1 has been determined by K. F. Bott (personal communication). Comparison of the two regions shows two identical sequences of about 80 nucleotides located downstream from the second promoter, the second one coding for the 5' end of the mature 16S ribosomal RNA. These data strongly support

the homology reported here. Interestingly, there are some differences in the region of the second promoter and the sequence containing the first promoter is absent in the ribosomal RNA genes set cloned in p14B1.

Within the 500-bp region, the *sar* sequence (suppressor for autonomous replication) has been found to be essential for the *cis*-inhibition of plasmid replication⁵. This sequence contains the two promoters described above. The elimination of the second promoter dramatically affected both the inhibitory effect and *in vitro* transcription, suggesting that the transcriptional activity of the second promoter is involved in the *cis*-inhibition of DNA replication⁵. The location of the *sar* sequence very close to or overlapping the replication origin could explain the failure to isolate an autonomous replicative sequence from the *B. subtilis* chromosome. The *sar* sequence has been assumed to control negatively the initiation of the chromosome and this effect should be removed during initiation. It is of particular interest to note the isolation of an RNA covalently linked to DNA and synthesized at specific times related to

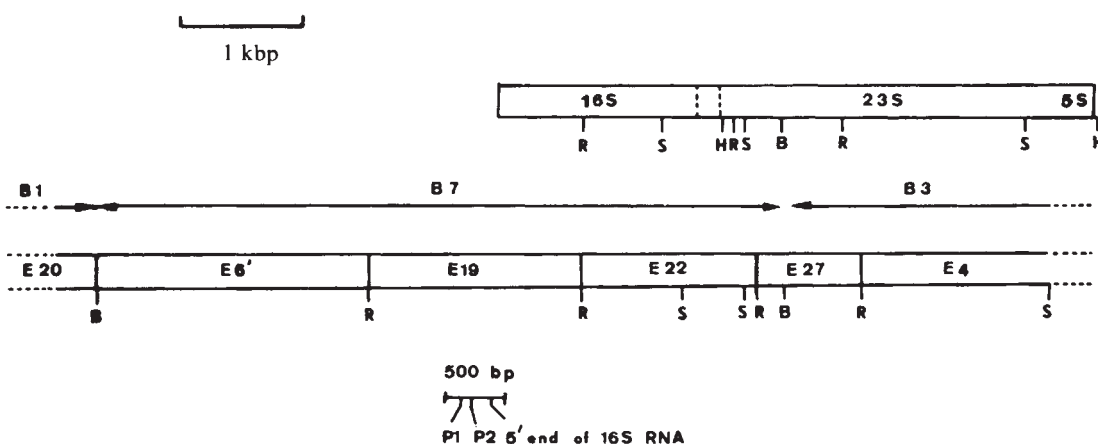


Fig. 4 Comparison of the restriction maps of the ribosomal RNA operon and the region of the replication origin. This figure also shows the location of the 500 bp sequenced segment of E19. P1 and P2 are the two promoters identified in the *sar* sequence. Data from Bott *et al.*⁹ and from Seiki *et al.*¹.

chromosome initiation⁹. Recently this RNA has been found to be homologous to the first replicating fragments E19 and E22 and thus appears as a possible candidate for the primer role at the replication origin (data not shown).

The chromosome replication origin has been located on the genetic map near *purA*, probably between *rec F15* and *ts132* loci¹². A DNA-protein complex which is known to be related to the cells' ability to initiate chromosome replication has been located near the origin, on the B1 fragment contiguous to the B7 fragment^{13,14}. In addition, the *gua* gene has been found by transformation to be present in the B3 fragment, which is adjacent to B7 (data not shown). The identification of a ribosomal RNA sequence on the first replicating fragment E19 provides more precise information on the organization of the replication origin region. From the results presented here, it can be concluded that the region covered by the two promoters and the 16S RNA sequences must contain the origin or be located within a few hundred nucleotides of the origin.

The ribosomal RNA genes are probably present in 10 copies in *B. subtilis*^{8,10,15} and some of these genes have been mapped^{16,17}. This study reports the location of a new ribosomal

RNA gene set, which we call *rrnO*, close to the replication origin. The promoters and the leading sequence of the *rrnO* gene set may be unique and responsible for the specific properties associated with the replication origin of the chromosome. This is the first indication in prokaryotes that ribosomal RNA cistrons may be located at the replication origin. However, studies on eukaryotic chromosomes indicate that replication origins occur in each repeating unit of the ribosomal RNA clusters¹⁸.

A major question in understanding the regulation of DNA replication is how the number of chromosomes is maintained in the cell at a certain value, through successive generations and through changes in the environment that alter growth rate. The finding in *B. subtilis*, of a ribosomal gene set in the region of the replication origin may throw light on this question.

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Abnormal NAD⁺ levels in cells from patients with Fanconi's anaemia

Nathan A. Berger, Sosamma J. Berger & Donna M. Catino

Hematology/Oncology Division, Departments of Medicine and Pharmacology, The Jewish Hospital of St Louis, and Washington University School of Medicine, St Louis, Missouri 63110, USA

Poly(ADP-ribose) is synthesized from NAD⁺ in the nuclei of eukaryotic cells and the activity of the chromatin-bound enzyme, poly(ADP-ribose) polymerase, is markedly stimulated by DNA strand breaks^{1–7}. Recent studies suggest that poly(ADP-ribose) is associated with the repair of DNA damage^{8–10} and the process of cellular differentiation^{11,12}. Hence, abnormalities in the metabolism of poly(ADP-ribose) could be aetiologically related to some of the human genetic disorders of DNA repair, especially those that are characterized by congenital and developmental anomalies. Defects in poly(ADP-ribose) metabolism could arise from abnormalities in the enzyme poly(ADP-ribose) polymerase, abnormalities in levels of its substrate NAD⁺, the proteins that act as acceptors for poly(ADP-ribose) polymerase or polymer stability. We have examined cells from patients with genetic disorders of DNA repair to determine whether they contain the enzyme poly(ADP-ribose) polymerase, whether the enzyme increases its activity in response to DNA damage and whether the cells maintain normal levels of NAD⁺. We demonstrate here that cells from patients having Fanconi's anaemia have lower NAD⁺ levels than cells from normal donors. This abnormality may contribute to the disorders of DNA repair and congenital anomalies that characterize this disease^{13,14}.

Table 1 shows the NAD⁺ levels found in a series of normal and repair-deficient human skin fibroblasts. The normal cells maintained a mean NAD⁺ level of 1,140 ± 304 pmol per 10⁶ cells with a range from 870 to 1,570 pmol per 10⁶ cells. Cells from patients in two of the subgroups of xeroderma pigmen-

tum maintained NAD⁺ levels that were slightly higher than normal. Cell lines from two patients with Cockayne's syndrome were examined; one had low and the other high NAD⁺ levels.

Cell lines derived from seven patients with Fanconi's anaemia (2061, 646, c111, 1196, c114, 368 and 1746) had low NAD⁺ levels compared with normal cells, while Fanconi's anaemia cell lines 2361, 369 and 449 maintained NAD⁺ levels that were in the low-normal range. The mean level of NAD⁺ in the Fanconi's fibroblasts was approximately half that of normal cells.

We then compared NAD⁺ levels in freshly prepared human lymphocytes derived from normal donors and from patients with Fanconi's anaemia. Normal human lymphocytes are resting intermitotic cells with low levels of pyridine nucleotides^{15,16}; when stimulated by phytohaemagglutinin (PHA), they undergo a marked expansion of their pyridine nucleotide pools, reaching peak levels on the third or fourth day of culture¹⁶. In the present study, NAD⁺ levels were measured in lymphocytes within 2–3 h of their preparation from fresh human blood and again after 3 days of PHA stimulation. Table 2 shows that the mean NAD⁺ level in resting lymphocytes from 35 normal donors was 80 ± 28 pmol per 10⁶ cells. Cells stimulated with PHA always showed marked increases in NAD⁺ levels, ranging from 3 to 16 times the NAD⁺ in resting cells.

Lymphocytes were prepared from five patients with Fanconi's anaemia and NAD⁺ levels were determined before and 3 days after PHA stimulation. Microscopic examination showed that the lymphocytes obtained from such patients underwent a normal agglutination reaction after PHA treatment. These cells also showed a normal increase in DNA synthesis as measured by ³H-thymidine (³H-TdR) incorporation on the third day after PHA stimulation. Initial NAD⁺ levels in freshly isolated lymphocytes from patients with Fanconi's anaemia were usually within the normal range. On PHA stimulation, the NAD⁺ levels increased; however, in the first four patients with Fanconi's anaemia, they were still below normal. Patient 4, whose PHA-stimulated lymphocytes exhibited an NAD⁺ level, at the lower limit of normal, was a 16-yr-old boy whose pancytopenia had partially responded to androgen therapy. Patient 5, who showed

Table 1 NAD⁺ levels (pmol per 10⁶ cells) in human fibroblasts

Normal cells		Xeroderma pigmentosum		Fanconi's anaemia	
Cell line	NAD ⁺	Cell line	NAD ⁺	Cell line	NAD ⁺
CRL1119	870 ± 247	CRL1223 (XPA)	2,210 ± 981	GM2061	245 ± 122
325	914 ± 281	CRL1160 (XPD)	1,470 ± 741	GM0646	286 ± 196
CRL1141	983 ± 217	Mean	1,840 ± 522	c111	398 ± 179
CRL1220	1,010 ± 361	Cockayne's syndrome		CRL1196	453 ± 107
CRL1121	1,480 ± 691			c114	512 ± 533
AG1518	1,570 ± 458			GM0368	699 ± 440
Mean	1,140 ± 304	Cell line	NAD ⁺	GM1746	789 ± 325
		GM0739	684 ± 363	GM2361	892 ± 540
		GM1856	2,290 ± 4	GM0369	907 ± 214
		Mean	1,490 ± 1,140	GM0449	973 ± 454
				Mean	614 ± 239

The sources of the human skin fibroblasts were as follows: AG1518, GM0739, GM1856, GM2061, GM0646, GM0368, GM1746, GM2361, GM0369 and GM0449 were all from the Human Genetic Mutant Cell Repository, Camden, New Jersey. CRL1119, CRL1141, CRL1220, CRL1121, CRL1223, CRL1160 and CRL1196 were from the American Type Culture Collection, Rockville, Maryland. Cell lines c111 and c114 were from Dr M. Swift, University of North Carolina. Cell line 325, from a patient with β -glucuronidase deficiency, was obtained from Dr W. Sly, Washington University Medical School. As these cells have no known disorder of DNA repair they are included in this series with the normal cell lines. All lines were maintained in alpha modified Eagle's medium supplemented with 20% heat-inactivated fetal calf serum (FCS) 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin and 25 mM HEPES, pH 7.2 at 37 °C. All studies were performed with three batches of FCS, both normal and abnormal cells were processed with each batch of serum and results were independent of the batch used. Most of the cell lines were examined for mycoplasma by independent assays at Flow Laboratories and all those examined were found to be free of contamination. For NAD⁺ determinations, confluent cultures in T75 flasks were split 1:3 into 100-mm Petri dishes with 15 ml medium and incubated at 37 °C. NAD⁺ assays were performed on the second day after plating. Cells were quickly scraped from plates, suspended in their growth medium by vortexing and the cell samples were processed for NAD determination by extraction and enzymatic cycling techniques as described previously^{16,24}. Additional samples of cell suspension were diluted into 5% Cetrimide and cell numbers counted electronically on a Coulter counter model F. All assays were performed in duplicate on duplicate samples taken at each time point. All assays were therefore performed in quadruplicate with less than 10% variation between separate assays. Results for each cell line are presented as the mean $\pm$ s.d. for assays performed at multiple passages between 7 and 20 except for cell lines CRL1220 and CRL1121 which were studied between passages 19 and 24. Differences between NAD⁺ values in normal and Fanconi's anaemia cells are significant at $P < 0.01$.

a completely normal increase in NAD⁺ in response to PHA, was a 20-yr-old woman whose pancytopenia was in a spontaneous partial remission. The mean NAD⁺ level in PHA-stimulated lymphocytes from all patients with Fanconi's anaemia was approximately half that detected in normal cells.

Table 2 NAD⁺ levels (pmol per 10⁶ cells) in human lymphocytes

	Control	PHA
Normal donors	80 ± 28	470 ± 180
FA patients		
1	59	176
2	27	66
	71	191
3	59	99
	74	244
4	39	252
5	98	693
Mean	61 ± 23	245 ± 208

Peripheral blood lymphocytes were prepared on Ficoll-Hypaque gradients as described previously^{16,17} from fasting normal donors and patients with Fanconi's anaemia. None of these donors were the same as those used in Table 1. The cells were diluted into alpha modified Eagle's medium supplemented with 2 mM glutamine, 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin, 10% heat-inactivated FCS and 25 mM HEPES, pH 7.2. For PHA studies, cells were incubated at 3×10^5 cells ml⁻¹ in a 20 ml T flask at 37 °C with 2 μ g ml⁻¹ sterile L-phytohaemagglutinin (Sigma). Control values were determined within 2–3 h of the time that cells were isolated from peripheral blood; PHA-stimulated values were determined after 3 days' incubation at 37 °C. At each time point cells were collected for NAD extraction and determination by enzymatic cycling assays as previously described^{16,24}. Additional samples were diluted into 5% Cetrimide for cell counting. All assays were performed in duplicate on duplicate samples taken at each time point; values shown are the means of assays performed in quadruplicate, with less than 10% variation between replicate assays. The five patients were all diagnosed as having Fanconi's anaemia (FA) based on pancytopenia, chromosomal abnormalities, typical skin changes, retarded growth and congenital anomalies of kidneys and bones. Their ages and sexes are as follows: 1, 10 yr (F); 2, 9 yr (F); 3, 11 yr (F); 4, 16 yr (M); 5, 20 yr (F). Patients 2 and 3 are sisters; cells from each of these two patients were examined on two separate occasions. Patient 4 was taking 2.5 mg oxymethalone every other day. The other patients were not receiving any drugs. Cells from 35 normal donors were used to establish the NAD⁺ level in control lymphocytes and cells from 25 of these donors were stimulated with PHA to determine the NAD⁺ levels in PHA-stimulated cells. There was no significant difference in NAD⁺ levels in resting lymphocytes between normal donors and patients with Fanconi's anaemia. The difference in NAD⁺ levels in PHA-stimulated cells between normal and Fanconi's anaemia patients was significant at $P < 0.01$.

As the highest NAD⁺ levels in the mitogen-stimulated lymphocytes from Fanconi's anaemia patients occurred in those two whose diseases were in partial remission, it is possible that their defect in maintaining NAD⁺ levels was improved. Alternatively, it is possible that the ability to achieve normal NAD⁺ levels contributes to the attainment of remission.

Additional studies were performed in which lymphocytes from normal donors were mixed with cells from patients with Fanconi's anaemia before extraction and measurement of NAD⁺ levels. The cells from patients with Fanconi's anaemia contained no substances that interfered with either the extraction or the measurement of NAD⁺. Furthermore, when lymphocytes from normal donors were co-cultivated with lymphocytes from Fanconi's anaemia patients, the final NAD⁺ values were those expected from the sum of the cells grown independently. Thus, Fanconi's anaemia cells contain no factors capable of reducing NAD⁺ levels in normal cells in the same culture system. Furthermore, the presence of normal cells does not correct the decreased NAD⁺ levels in the Fanconi's anaemia cells.

Freshly prepared human lymphocytes were also used to assay for the presence of poly(ADP-ribose) polymerase in Fanconi's anaemia and to determine whether its activity increased in a normal manner in response to DNA damage¹⁷. These studies were performed in lymphocytes that were untreated (control cells), or treated either with UV irradiation (50 J m⁻²) or with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; 20 μ g ml⁻¹), incubated for 3 h at 37 °C, then permeabilized and supplied with ³H-NAD to measure the activity of poly(ADP-ribose) polymerase¹⁷. Table 3 shows that the enzyme was present and its activity increased in normal cells in response to treatment with UV irradiation or MNNG. Similarly, it was present in cells from patients with Fanconi's anaemia and its activity increased in response to UV irradiation and treatment with MNNG. While the latter cells showed less enzyme activity than the normal cells, they nevertheless did respond to DNA damage as did normal cells with an increase in activity of poly(ADP-ribose) polymerase.

As a result of the increase in poly(ADP-ribose) polymerase activity that occurs in response to DNA damage, cellular pools

Table 3 Poly(ADP-ribose) synthesis (pmol per 10^6 cells per 30 min) in DNA-damaged lymphocytes

	Normal donors	Donors with Fanconi's anaemia
Control	36 ± 18 (n = 15)	17 ± 4 (n = 2)
UV-irradiated (50 J m ⁻²)	179 ± 70 (n = 12)	90 ± 0 (n = 2)
MNNG (20 µg ml ⁻¹)	511 ± 235 (n = 5)	260 ± 171 (n = 2)

Immediately after preparation of peripheral blood lymphocytes from normal donors or patients with Fanconi's anaemia, the cells were suspended at 3×10^5 ml⁻¹ in the same medium as described in Table 2. Control cells received no further treatment. UV-irradiated cells received 50 J m⁻² as previously described¹⁷ and MNNG-treated cells were adjusted to contain final concentrations of 20 µg ml⁻¹ MNNG and 0.2% dimethyl sulphoxide (DMSO). Previous studies have shown that mock-irradiation or DMSO treatment has no effect on poly(ADP-ribose) synthesis²⁰. Cells were incubated in tissue culture medium at 37 °C for 3 h, then permeabilized; 10^6 cells were then incubated with ³H-NAD (NEN) for 30 min and treated with 20% cold trichloroacetic acid to measure incorporation into poly(ADP-ribose) as described previously¹⁷. The result for each donor is the mean of assays performed in triplicate which agreed within 10%. The results for normal cells are expressed as the mean ± s.d. of assays performed on cells from the number of donors is indicated in parentheses. The values for the patients with Fanconi's anaemia were obtained for lymphocytes from two donors, patients 1 and 5 in Table 2.

of NAD⁺ can be rapidly consumed¹⁸⁻²¹. Table 4 shows that NAD⁺ levels decreased in normal and Fanconi's anaemia cells after UV irradiation and treatment with MNNG. This fall in NAD⁺ levels serves as an independent demonstration that DNA damage activates poly(ADP-ribose) polymerase in Fanconi's anaemia cells as it does in normal cells^{20,21}. Although these determinations were only performed on cells from two donors, it is interesting that the residual NAD⁺ levels in Fanconi's anaemia cells were lower than in normal cells. These exceptionally low NAD⁺ levels may severely impair the ability of Fanconi's anaemia cells to maintain their energy metabolism during the period required for the normal DNA repair processes. For example, when the NAD⁺ levels of L1210 and 3T3 cells are depleted by growth in nicotinamide-deficient medium, they lose their ability to perform unscheduled DNA synthesis and to reseat DNA strand breaks^{8,9}. Thus, the low NAD⁺ levels in cells from some patients with Fanconi's anaemia may contribute to their decreased ability to recover from DNA damage.

Many studies have identified NAD⁺ levels and poly(ADP-ribose) synthesis as important factors in the normal process of differentiation. Nicotinamide analogues that interfere with NAD⁺ synthesis prevent normal differentiation and result in congenital anomalies in developing embryos²². Incubation of chick mesenchymal cells in high levels of nicotinamide induce high intracellular NAD⁺ levels and cause cells to differentiate into myoblasts, whereas incubation in medium with low nicotinamide result in NAD⁺-depleted cells and cause differentiation into cartilage cells¹¹. Poly(ADP-ribose) polymerase activity increases when myoblasts differentiate into myotubes and when Friend erythroleukaemic cells are induced to synthesize haemoglobin^{12,23}. Thus, the pleiotropic abnormalities that occur in patients with Fanconi's anaemia may be explained by abnormalities in the sizes of their NAD⁺ pools.

More studies are clearly required to determine whether decreased NAD⁺ pools in patients with Fanconi's anaemia are due to underproduction or over-utilization. In addition, it will

Table 4 NAD⁺ levels (pmol per 10^6 cells) in DNA-damaged lymphocytes

	Normal donors	Donors with Fanconi's anaemia
Control	79 ± 20 (n = 19)	80 ± 28 (n = 2)
UV-irradiated (50 J m ⁻²)	11 ± 7 (n = 15)	0.7 ± 0.1 (n = 2)
MNNG (20 µg ml ⁻¹)	6 ± 7 (n = 6)	0.4 ± 0.5 (n = 2)

Control and DNA-damaged cells were treated as described in Table 3 then incubated in tissue culture medium at 37 °C for 3 h. Cells were collected for NAD assays and cell counts as described in Table 2. The values represent the mean ± s.d. of assays performed on cells from the number of donors given in parentheses. The assays using cells from patients with Fanconi's anaemia were performed on lymphocytes from two donors, patients 1 and 5 in Table 2.

be necessary to determine whether the low NAD⁺ levels are the cause or consequence of the DNA repair defect. Finally, longitudinal studies will be required to investigate the relationship of NAD⁺ levels to the severity of the disease and the possibility that some of the abnormalities may be ameliorated by correcting NAD⁺ levels.

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Sulphation of tyrosine residues—a widespread modification of proteins

Wieland B. Huttner

Department of Neurochemistry, Max-Planck-Institute for Psychiatry, D-8033 Martinsried/Munich, FRG

Of the many known covalent modifications of proteins¹, few have been recognized as widespread. Among these modifications, protein phosphorylation has been extensively studied²⁻⁴. Recently, phosphorylation of tyrosine residues has been implicated in regulatory events such as cell transformation and hormone-induced cell growth⁵⁻⁷. The present study explores the possibility that another modification of tyrosine residues in proteins, tyrosine sulphation, may be widespread. This modification is particularly interesting because of the molecular resemblance of tyrosine-O-sulphate and tyrosine-O-phosphate. Protein sulphation on tyrosine residues was found to occur in all cell types in culture and all tissues *in situ* so far examined. Moreover, for each cell type and tissue, proteins containing tyrosine-O-sulphate were found in distinct electrophoretic patterns throughout the molecular weight range studied. Thus, protein sulphation on tyrosine residues appears to be a widespread covalent modification, and may have an important role in cell function.

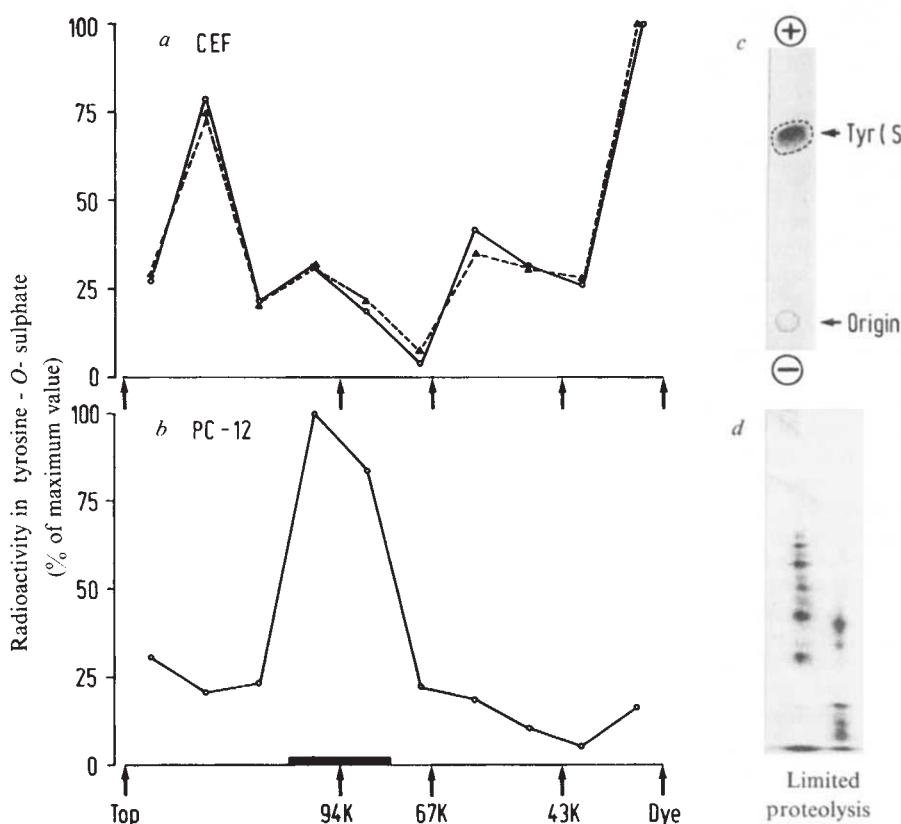
Protein sulphation on tyrosine residues was studied in the following cells in culture: bovine adrenal cells, chicken embryo fibroblasts, chicken embryo muscle cells, chicken embryo retina cells, rat brain cells and rat pheochromocytoma cells. Cell cultures were incubated with either ³⁵S-sulphate or ³⁵S-sulphate plus ³H-tyrosine (see Fig. 1 legend for experimental details).

Cellular proteins were then separated by SDS-polyacrylamide gel electrophoresis. The gels were divided from the top to the dye front into 10 equal portions containing the cellular proteins of high molecular weight to low molecular weight, respectively. Proteins from the individual gel pieces were hydrolysed, and the hydrolysates were analysed for radioactive tyrosine-*O*-sulphate by thin-layer electrophoresis at pH 3.5 followed by autoradiography. A typical autoradiogram of ^{35}S -labelled material obtained from one of the gel pieces is shown in Fig. 1c. The following lines of evidence, taken together, indicate that the radioactive substance migrating towards the anode on electrophoresis at pH 3.5 was indeed tyrosine-*O*-sulphate. (1) The radioactive substance migrated exactly as authentic tyrosine-*O*-sulphate⁸ in this one-dimensional separation (Fig. 1c) and on two-dimensional electrophoresis at pH 1.9 and pH 3.5. The migration of the radioactive substance was clearly different from that of methionine and cysteine (neither of which migrated towards the anode at pH 3.5) and from that of cysteic acid (which migrated ~1.5 times faster towards the anode than tyrosine-*O*-sulphate). (2) The radioactive substance appeared to be an amino acid since it could also be generated by extensive pronase digestion alone. (3) The radioactive substance appeared to have the same pH-stability⁹ as authentic tyrosine-*O*-sulphate since the sulphate group was not removed by alkali treatment but was removed by treatment with 1 M HCl for 5 min at 100 °C. (4) The radioactive substance, when obtained from double-labelled cells, contained both ^{35}S -sulphate and ^3H -tyrosine (Fig. 1a).

The radioactivity in the tyrosine-*O*-sulphate spot obtained after thin-layer electrophoresis of the protein hydrolysates was quantitated for all 10 gel pieces of each cell type studied in culture. The electrophoretic patterns of protein-incorporated radioactive tyrosine-*O*-sulphate thus obtained are illustrated in Fig. 1 for chicken embryo fibroblasts (CEF, panel a) and rat pheochromocytoma cells (PC-12, panel b). For both CEF and PC-12 cells, proteins that had incorporated ^{35}S -sulphate on tyrosine residues were found in all gel pieces, that is throughout the molecular weight range studied. However, the electrophoretic pattern of protein-incorporated ^{35}S -tyrosine-*O*-sulphate observed for CEF (Fig. 1a, solid line) was clearly different from that observed for PC-12 cells (Fig. 1b, solid line). Since the CEF proteins had been double-labelled with both ^{35}S -sulphate and ^3H -tyrosine, it was also possible to analyse the sulphation of tyrosine residues by counting the tyrosine-*O*-sulphate spot for ^3H -label present on the benzene ring of tyrosine. The electrophoretic pattern of protein-incorporated ^3H -tyrosine-labelled tyrosine-*O*-sulphate (Fig. 1a, dashed line) was found to be almost identical to that of ^{35}S -sulphate-labelled tyrosine-*O*-sulphate (Fig. 1a, solid line). Using the experimental approach illustrated in Fig. 1, the other cell types studied in culture (adrenal, brain, muscle and retina cells) also gave distinct electrophoretic patterns of cellular proteins containing tyrosine-*O*-sulphate throughout the molecular weight range studied.

In the analysis of PC-12 cells (Fig. 1b), the 120K–80K molecular weight region of the gel (indicated in Fig. 1b by the

Fig. 1 Protein sulphation on tyrosine residues in chicken embryo fibroblasts (CEF) and rat pheochromocytoma cells (PC-12) in culture. Cells were incubated for 18 h in sulphate-free Dulbecco's modified Eagle's medium (DMEM) containing 0.3 mCi ml⁻¹ of ^{35}S -sulphate and, in the case of CEF, 25 µCi ml⁻¹ of L-[3, 5- ^3H]tyrosine. (The concentration of 'cold' sulphate in normal DMEM was sufficient to reduce radioactive sulphate incorporation markedly.) The medium was then removed, the cells were solubilized in Laemmli sample buffer¹⁶, and cellular proteins separated on a 7.5% SDS-polyacrylamide gel. The gel was fixed in 50% methanol/10% acetic acid, stained with Coomassie blue, and dried. Each lane of the dried gel, containing either CEF or PC-12 cell proteins, was then cut from the top to the dye front into 10 equal portions representing different molecular weight regions. The individual gel pieces were reswollen in 50 mM ammonium bicarbonate containing 100 µg ml⁻¹ of pronase and incubated for 24 h at 37 °C. The eluates were lyophilized, dissolved in 0.2 M barium hydroxide, evacuated, incubated for 24 h at 110 °C, neutralized with sulphuric acid, and centrifuged. The supernatants were lyophilized, dissolved in a solution containing 5 µg of tyrosine-*O*-sulphate standard⁸, spotted on TLC-cellulose sheets, and subjected to electrophoresis in 5% acetic acid/0.5% pyridine, pH 3.5⁵. Panel c shows a typical autoradiogram after thin-layer electrophoresis of ^{35}S -labelled material obtained from one of the gel pieces. The dashed line indicates the position of the tyrosine-*O*-sulphate standard, detected by ninhydrin staining. For each of the 10 gel pieces of CEF (panel a) and PC-12 cells (panel b), the radioactivity in the tyrosine-*O*-sulphate spot was determined by liquid scintillation counting and is expressed as per cent of that obtained from the gel piece giving the maximum value (100%): ^{35}S (solid lines) CEF 3,925 c.p.m., PC-12 cells 2,405 c.p.m.; ^3H (dashed line) CEF 294 c.p.m. Values are from left to right for gel pieces containing high molecular weight proteins to gel pieces containing low molecular weight proteins. Arrows indicate the positions of molecular weight marker proteins. The solid bar on the abscissa of panel b indicates the region of the gel that was subjected to peptide mapping after limited proteolysis¹⁰ (1.5 µg of *Staphylococcus aureus* V8 protease) with the modifications described elsewhere¹⁶. Panel d shows an autoradiogram of the proteolysis gel.



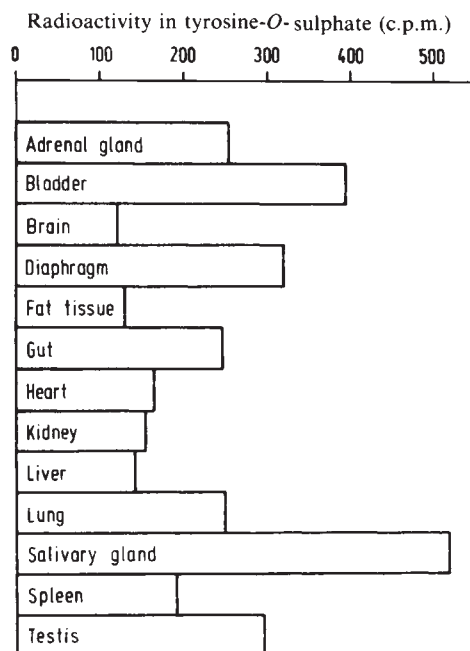


Fig. 2 Protein sulphation on tyrosine residues in various rat tissues *in situ*. At 18 h after an intraperitoneal injection of 20 mCi of ^{35}S -sulphate, rat tissues were freed of blood by perfusion with saline and were then rapidly homogenized in 3% SDS. Aliquots of each tissue containing equal amounts of protein (300 μg) were subjected to SDS-polyacrylamide gel electrophoresis and analysed for protein-incorporated ^{35}S -tyrosine-*O*-sulphate as described in Fig. 1 legend. The radioactivity in tyrosine-*O*-sulphate is given for tissue proteins from a whole lane of the gel.

solid bar) contained the highest amounts of protein-incorporated ^{35}S -tyrosine-*O*-sulphate. The same region of a duplicate gel was subjected to limited proteolysis¹⁰ during a second SDS-polyacrylamide gel electrophoresis step. Subsequent autoradiography of the proteolysis gel showed that the proteins from the 120K–80K region yielded two distinct patterns of ^{35}S -labelled fragments (Fig. 1d). Extensive pronase treatment of individual gel pieces containing these fragments yielded ^{35}S -tyrosine-*O*-sulphate in each case.

Since sulphation of proteins on tyrosine residues occurred in a variety of cell types in culture, it was of interest to determine whether this modification could also be observed after *in vivo* labelling of tissues *in situ*. For this analysis (see Figs 2 and 3 legends for experimental details), 20 tissues were dissected from a rat 18 h after the animal had been injected intraperitoneally with inorganic ^{35}S -sulphate. With a single injection of 20 mCi of inorganic ^{35}S -sulphate into a 150 g rat, one would expect a marked dilution of the radioactive label and a great reduction in its specific activity because of the presence of endogenous inorganic sulphate in the body fluids. Nevertheless, radioactive sulphate incorporation into proteins on tyrosine residues could be detected in all 20 rat tissues examined. The results for 13 tissues are given in Fig. 2.

In addition, the electrophoretic pattern of proteins containing ^{35}S -tyrosine-*O*-sulphate was also determined for each tissue, using the experimental approach described above for cultured cells. These results are illustrated in Fig. 3 for three of the tissues examined, adrenal gland, gut and lung. These three tissues contained similar amounts of the total protein-incorporated ^{35}S -tyrosine-*O*-sulphate. However, the electrophoretic patterns of tissue proteins containing ^{35}S -tyrosine-*O*-sulphate were distinct for the three tissues. In fact, all the other tissues examined also gave distinct electrophoretic patterns of proteins containing ^{35}S -tyrosine-*O*-sulphate throughout the molecular weight range studied.

Only one protein, fibrinogen⁹, and a few biological peptides^{11–15} have so far been demonstrated to contain tyrosine-*O*-sulphate. The results of this study provide evidence that in all

cell types in culture and all tissues *in situ* so far examined, many proteins are sulphated on tyrosine residues. Two observations could help to explain why this widespread occurrence of protein sulphation on tyrosine residues has not been reported so far. First, the ester bond of tyrosine-*O*-sulphate is remarkably acid-labile⁹. The sulphate group is therefore likely to be hydrolysed in some of the common protein-chemical procedures. Second, ^{35}S -sulphation of proteins and other macromolecules on carbohydrate moieties tends to obscure the presence of proteins containing ^{35}S -tyrosine-*O*-sulphate, for example on autoradiography of SDS-polyacrylamide gels.

The widespread occurrence of protein sulphation on tyrosine residues suggests that this modification may have an important role in cell function. The distinct electrophoretic patterns of proteins containing tyrosine-*O*-sulphate that were observed in many different cell types raise the possibility that protein sulphation on tyrosine residues may be involved in a variety of cellular processes.

If protein sulphation on tyrosine is found to occur in the same subcellular compartment as phosphorylation on tyrosine, then the possibility should be considered that tyrosine residues which are physiologically *O*-sulphated may be subject to 'false' modification by becoming *O*-phosphorylated (and vice versa). Such false modification may have significant biological consequences. The false modification of tyrosine residues may not be fully recognized as such by the cell, since there is a molecular resemblance of tyrosine-*O*-sulphate and tyrosine-*O*-phosphate. The false modification of tyrosine residues may, however, be dissimilar enough from the physiological modification to perturb the balance of cellular regulation.

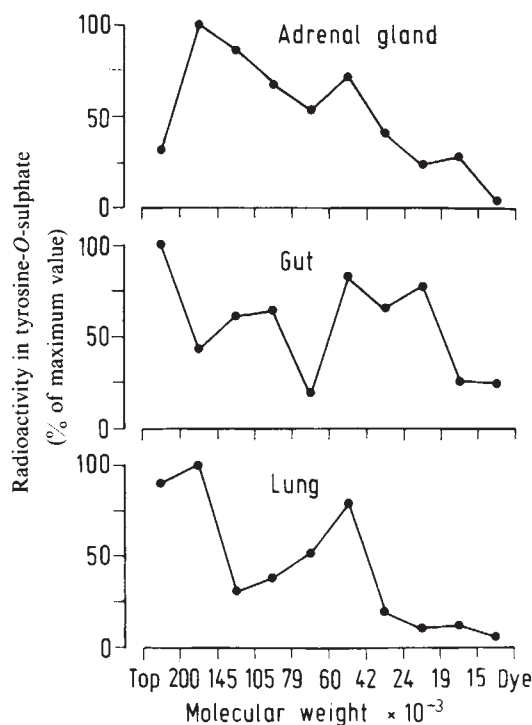


Fig. 3 Electrophoretic patterns of proteins containing tyrosine-*O*-sulphate in rat adrenal gland, gut and lung. The tissues were labelled *in situ* with ^{35}S -sulphate and tissue proteins were subsequently solubilized as described in the legend to Fig. 2. Aliquots containing equal amounts of proteins (3 mg) were subjected in duplicate to SDS-polyacrylamide gel electrophoresis on either 7.5% or 15% gels. Each of the gels was cut into five portions, representing different molecular weight ranges as indicated on the abscissa. Gel pieces containing molecular weight ranges above 60,000 were obtained from the 7.5% gels, the other gel pieces were obtained from the 15% gels. Analysis of individual gel pieces for protein-incorporated ^{35}S -tyrosine-*O*-sulphate was performed as described in the legend to Fig. 1. Maximum values (100%) were: adrenal gland 583 c.p.m., gut 477 c.p.m., lung 616 c.p.m.

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Diversity of cholesterol exchange explained by dissolution into water

Eigil Bojesen

Institute of Experimental Hormone Research, University of Copenhagen, 71 Nørre Alle, DK-2100 Copenhagen, Denmark

It is usually considered that the stability of lipid bilayers results from the elastic resistance of water to the dissolution of hydrophobic compounds^{1,2}. However, attempts to describe this unique property of water in more detail^{2,3} have been difficult and some authors^{4–7} believe that the dispersion attraction of apolar molecules (London–van der Waals forces) is the most important factor. Details of the transfer kinetics into water of apolar compounds may elucidate this. Here I describe an analysis of the kinetics of cholesterol exchange between erythrocytes and lipoproteins that makes it possible to calculate precisely the widely differing specific rate constants of cholesterol dissolution from both kinds of structures. The analysis also accounts qualitatively for the different exchange kinetics reported for homologous and heterologous dispersed systems in water.

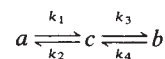
The fairly rapid exchange of cholesterol between erythrocytes and lipoproteins⁸ was originally assumed to reflect the dissolution of cholesterol from both dispersed phases. However, this has been questioned as the rate of exchange in this and other heterologous binary systems depends on the concentrations of the lipid particles^{9–12}, suggesting a collision frequency limitation, and transfer during collision. However the surfaces of lipoproteins and erythrocytes in rats are, on average, more than 100 Å apart¹³, closer contact being prevented by the cell glycocalyx. Furthermore, the kinetics of cholesterol exchange in pure homologous binary systems of artificial lipid vesicles is incompatible with a collision limitation^{14–16}. The problem remains of why the kinetics are so different in heterologous and homologous systems.

Consider a closed system containing the amounts *A* and *B* of cholesterol in two dispersed lipid phases in equilibrium. If the exchange of cholesterol between the two phases is mediated solely by the amount, *C*, of cholesterol in water the scheme of equilibrium becomes:



In a homologous system *A* and *B* could be present in two separable types of liposomes and in a heterologous system, such as erythrocytes in serum, they represent the cholesterol of the cell membranes and in the lipoproteins, respectively.

In the tracer experiments in which *A* or *B* is labelled initially, the most simple corresponding scheme for the time-dependent amounts of *a*, *b* and *c* of tracer is



In this scheme it is assumed that only one pair of rate constants describes the transfer of the tracer in and out of each of the dispersed phases. As *c* is very small compared with *a* and *b* the scheme describes an experiment with monophasic exchange kinetics eventually leading to isotopic equilibrium. As such it does not differ from a simple two pool model, but whereas the two pool model is only a mathematical concept, the above scheme includes rate constants of real physical significance. As the processes are slow compared with diffusion equilibria within distances of ~100 Å, the tracer is evenly distributed in the aqueous phase with the concentration *c_w*. If α and β represent the reactive surface areas of the two kinds of dispersed phases the system approaches tracer equilibrium according to the two rate equations

$$-da/dt = k_1a - k_2c_w\alpha \quad (1)$$

$$-db/dt = k_3b - k_4c_w\beta \quad (2)$$

Thus *k₁* and *k₃* are first order whereas *k₂* and *k₄* are second order rate constants.

By eliminating *c_w* and using the substitution

$$Q = \frac{k_2\alpha}{k_4\beta}$$

we get

$$da/dt + k_1a = Q(db/dt + k_3b) \quad (3)$$

At equilibrium, when $t \rightarrow \infty$, the ratio a_∞/b_∞ is determined by

$$\frac{a_\infty}{b_\infty} = Q \frac{k_3}{k_1} = f_\infty \quad (4)$$

The low water solubility of cholesterol¹⁷ means that the total amount of tracer, *R*, in the system is equal to (*a* + *b*) and that $da/dt = -db/dt$.

Thus one of the two variables in (3) can be eliminated. Written in terms of *a* we get

$$da/dt + k_1a = -Qda/dt + k_3QR - k_3Qa \quad (5)$$

As *Q* and *R* are time-independent this equation can be solved by separation of the variables, followed by integration between the limits of *t* from 0 to *t* and of *a* from *a₀* to *a_t*. Using the identities (*R* - *a₀*) = *b₀*, (*R* - *a_t*) = *b_t*, and $Q = f_\infty(k_1/k_3)$, we get

$$\ln \left[\frac{a_t - f_\infty b_t}{a_0 - f_\infty b_0} \right] = -t \left[\frac{k_1(1 + f_\infty)}{1 + (k_1/k_3)f_\infty} \right] \quad (6)$$

This equation defines a rate constant ε

$$\varepsilon = \frac{k_1(1 + f_\infty)}{1 + (k_1/k_3)f_\infty} \quad (7)$$

Table 1 The specific rate constants of cholesterol dissolution from red cells and lipoproteins

Type of experiment	No. of experiments	Correlation coefficient	<i>k₃</i> (h ⁻¹) (mean ± s.e.m.)	<i>k₁</i> (h ⁻¹) (mean ± s.e.m.)
Cell → Serum	18	0.988	3.10 ± 0.12	0.224 ± 0.012
Serum → Cell	24	0.985	3.30 ± 0.12	0.203 ± 0.007
Both types, all <i>F</i>	42	0.986	3.21 ± 0.087	0.211 ± 0.006
Both types (1.15 < <i>F</i> < 5.8)	25	0.70	2.82 ± 0.61	0.225 ± 0.014

k₁ and *k₃* are the rate constants for dissolution of cholesterol from red cells and from lipoprotein, respectively, calculated on the basis of the linear regression $(1 + F)/\varepsilon = F/k_3 + 1/k_1$.

Fig. 1 a. The time constant (h^{-1}) of the exchange of cholesterol at 37°C between rat erythrocytes and serum lipoproteins as a function of the cell: cholesterol pool ratio F . ●, Results of cells $\rightarrow$ serum assays; ○, results of serum $\rightarrow$ cells assays. F values < 9 were obtained by variation of the haematocrit whereas higher values required dilution of serum. A special washing procedure¹³ was applied in order to preserve the disk shape of cells isolated from EDTA blood of fasted rats. The final medium was an oxygenated 1:1 mixture of isotonic sucrose and a slightly hypotonic 2 mM phosphate buffer with 5 mM glucose and 1 mM CaCl_2 , pH 7.4. Serum from the same rat was heated at 50°C for 45 min to block ester formation and then labelled with $\sim 0.5 \mu\text{Ci}$ purified ^3H cholesterol (specific activity 50 Ci mol^{-1} ; Amersham) deposited on small glass beads²⁵. 4 ml of serum were labelled and used directly in the serum $\rightarrow$ cells assays. 1 ml of labelled serum diluted twofold with the sucrose buffer medium was incubated for 2 h with 2.5 ml of packed cells for cells $\rightarrow$ serum assays.

The labelled cells were stored at 5°C overnight in the sucrose buffer medium after numerous washings. The assay was made with a vigorously shaken 2 ml cell suspension in serum or diluted serum with a 5% CO_2 atmosphere. The time intervals between collections of 6 or 7 aliquots (200 μl) were increased progressively to approach a linear change in fractional equilibria from < 0.1 to > 0.9 . After quantitative separation of cells from the serum by centrifugation and washing, the lipids were extracted after haemolysis and dilution of the two fractions. The extracts were used either for tracer measurements, liquid scintillation counting, or for chemical analyses of cholesterol, separated quantitatively from cholesteryl esters by column chromatography²⁵. Two additional aliquots per assay were used for this purpose. The time constant and tracer equilibrium for the assays were computed as described in the text. The curve shows the hyperbolic relationship between ϵ and F according to

$$\epsilon = \frac{k_1(1+F)}{1 + (k_1/k_3)F}$$

with k_1 and k_3 of Table 1 computed on the basis of all the 42 assays. Also, the two asymptotes are drawn: $\epsilon = k_3$ and $F = -k_3/k_1$. **b** Shows the same data plotted according to the linear regression $1 + F/\epsilon = F(1/k_3) + 1/k_1$. The drawn regression line is $y = x(0.312 \pm 0.0084) + (4.74 \pm 0.143)$.

In a homologous system ϵ is independent of f_∞ , as $k_1 = k_3$; however ϵ is a function of f_∞ in heterologous systems, for which $k_1 \neq k_3$. Equation (7) describes an equilateral hyperbola, with $\epsilon = k_3$ and $f_\infty = -(k_3/k_1)$ as the asymptotes. The equation can be transformed into

$$\frac{1+f_\infty}{\epsilon} = f_\infty/k_3 + 1/k_1 \quad (8)$$

and k_1 and k_3 can therefore be obtained by a linear regression analysis of (ϵ, f_∞) data, using a suitable wide range of f_∞ values.

For the system of erythrocytes in serum we assign A and a to the cells, and B and b to the lipoproteins. With the cells initially labelled (cells $\rightarrow$ serum assays) we have $a_0 = R = (a_i + b_i)$, and $b_0 = 0$. The specific rate constants of tracer dissolution, k_1 and k_3 are then assigned to the cells and the lipoproteins, respectively. In such assays, performed as described in Fig. 1 legend, the two parameters ϵ and f_∞ were computed from the b_i or $f_i = a_i/b_i$ values of a time series of aliquots. Two transforms of the left-hand side of (6) were used: $\ln(1 - b_i/b_\infty)$ or $\ln[(f_i - f_\infty)/(1 + f_i)]$. From computed values of b_∞ one immediately gets f_∞ , as $f_\infty = (R/b_\infty - 1)$. The optimal values of f_∞ (or b_∞) were obtained as the values which in the regression analysis resulted in a minimum of standard error of the time constant. The left-hand side of (6) was weighted according to the computed variances¹⁸, assuming that the coefficient of variation in the determination of f_i or b_i is equal to that of the total counts of the aliquots (3%).

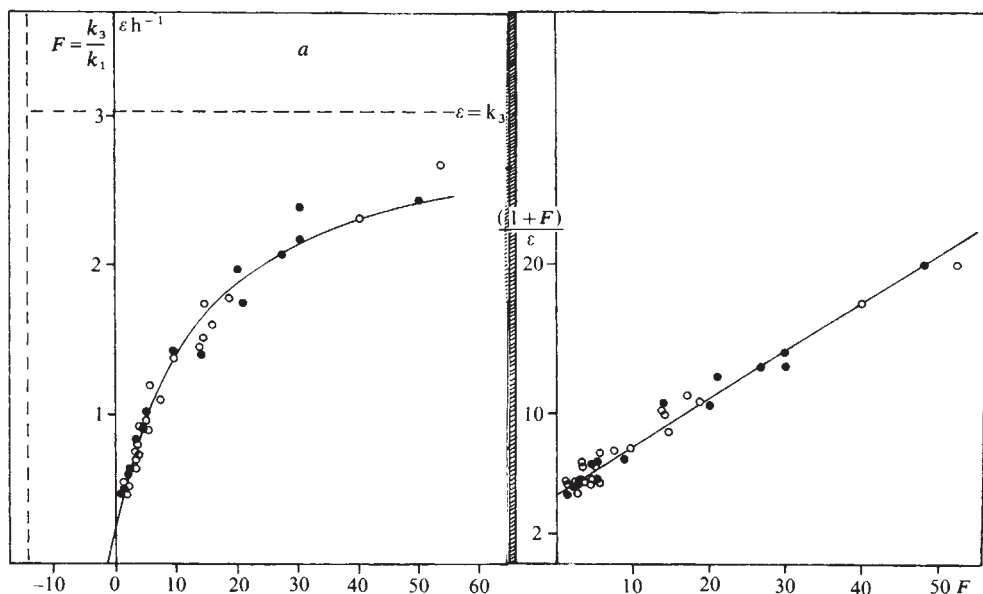
This analysis has demonstrated the validity of the basic assumptions expressed in equations (1) and (2). The exchange kinetics were monophasic, within the analytical error as the data always defined the rate constant ϵ with a coefficient of variation smaller than 4% and a numerical value of the intercept of the regression line smaller than ± 0.04 . Furthermore, the regression line describes the approach to complete tracer equilibrium. The mass ratio of cholesterol, $F = A/B$, obtained by chemical analysis of cholesterol^{13,19}, and the computed f_∞ were identical, within analytical errors ($f_\infty/F = 1.001 \pm 0.045$ ($n = 14$)). Therefore F can replace f_∞ in equations (7) and (8).

In the reverse experiments (serum $\rightarrow$ cell assays) only the

tracer uptake was used in the computation of ϵ . The procedure for labelling the lipoproteins resulted directly in the formation of a small amount of unexchangeable tracer ($< 10\%$) and therefore in erroneous f_∞ values. Thus only the chemically determined F is available for this type of assay.

The results obtained at 37°C for both types of assays are presented in Fig. 1a; the same data, plotted according to equation (8) are shown in Fig. 1b. This linear regression was used to calculate the specific rate constants k_1 and k_3 as the scatter of the 42 points of $(F, (1-F)/\epsilon)$ does not vary with F . The coefficients of variation of the reciprocal values of the two constants were obtained directly and used to calculate the s.e.m. in the estimation of rate constants. Table 1 shows the results of this analysis for all the 42 data points covering the whole range of F values and for the 18 cells $\rightarrow$ serum as well as the 24 serum $\rightarrow$ cells experiments separately. Clearly it makes no difference whether the initially labelled partner is serum or cells. Table 1 also shows that assays with F confined to the lower range give a well defined value for k_1 but a crude value for k_3 which is not significantly different from that calculated on the basis of all the data. The result that k_3 was much larger than k_1 was expected. According to some authors^{20,21} the hydroxyl group of cholesterol is hydrogen-bonded to the carbonyl group of lecithin, and others^{22,23} have observed that the mobility of cholesterol is less restricted in the lipoproteins. The observation that k_3 has a well defined value is perhaps surprising, considering the chemical diversities of lipoproteins. The observed monophasic exchange kinetics and the identity of the calculated f_∞ with F over the whole range of F values demonstrate, however, that the variation in k_3 within lipoproteins must be relatively small. However, the sensitivity of this criterion should not be overrated²⁴ and assays with separated lipoproteins are needed.

Equation (7) predicts that the time constant of exchange is only independent of F if $k_1 = k_3$. This is in accordance with the difference in the kinetics of homologous and heterologous systems as referred to above. If k_1 is much smaller than k_3 in heterologous systems the term $(k_1 F)/k_3$ vanishes provided the two dispersed phases contain about equal amounts of



cholesterol. The time constant of exchange then varies in proportion to $(1+F)$, and approaches the limiting value of k_1 as F approaches zero. This prediction is again, at least qualitatively, in agreement with reported observations^{9,10,12}.

Thus the phase partition theory⁸ accounts for the exchange kinetics of homologous artificial systems as well as for the natural heterologous system in blood. The idea of transfer by collision is therefore redundant. Since the specific rate constants

k_1 and k_3 can be estimated quite accurately (Table 1) the effect of temperature will provide information on the energy by which cholesterol and probably other apolar compounds are bound in different dispersed phases.

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Two genes for threonine accumulation in barley seeds

Simon W. J. Bright, Joseph S. H. Kueh,
Julian Franklin, Sven Erik Rognes*
& Benjamin J. Mifflin

Biochemistry Department, Rothamsted Experimental Station,
Harpenden AL5 2JQ, UK

* Botanical Institute, University of Oslo, Blindern, Oslo 3, Norway

Cereal seed protein is the major direct source of dietary protein for man and also serves as an indirect source through the feeding of livestock. For non-ruminant animals, such as man, swine and poultry, most cereal seed proteins are deficient in lysine and threonine and much effort has been devoted to their improvement¹. Supplementation of barley diets for pigs with lysine and threonine is sufficient to produce a feed with protein of high biological value². Mutant lines that accumulate these amino acids in the seed could therefore be of agricultural value. The synthesis of lysine, threonine and methionine, which are all derived from aspartic acid, is regulated in plants by a series of feedback loops^{3,4}. This regulation is such that when barley embryos are germinated on a medium containing lysine plus threonine (LT medium) the plants are starved of methionine and fail to grow⁵. Plants which do grow in these conditions may contain enzymes that are no longer feedback-regulated and that may allow enhanced amounts of end-product amino acids to accumulate. We report here the identification of two genes in barley, mutations in which lead to decreased feedback sensitivity of the enzyme aspartate kinase, resistance to lysine plus threonine and, in some cases, to accumulation of soluble threonine in the seed.

From an M_2 population of 10^4 mature embryos (M_1 treated with sodium azide) three mutants resistant to lysine plus threonine (2–3 mM) were selected for further study. These were R(Rothamsted) 2501 (ref. 6), R3004 and R3202 which produced sensitive and resistant progeny in the M_3 generation. Pure-breeding resistant lines were obtained from R3004 and R3202 and, with difficulty, from R2501. The designation R3004 or R3202 refers henceforth to the M_3 and subsequent generations of such lines.

Both R3202 and R3004 plants grew better than controls in a range of concentrations of lysine and threonine (Fig. 1). R3202 plants were generally larger and had better root growth than R3004. Both mutant lines had lost the synergistic inhibition of growth by lysine plus threonine (Fig. 2) but were still slightly inhibited by lysine alone. This was reduced by addition of arginine. Neither R3202 nor R3004 were resistant to the lysine analogue S-(2-aminoethyl)cysteine.

To characterize the genetic basis of resistance, mutant plants were crossed among themselves and with normal barley cv. Golden Promise. Crosses with Golden Promise (Table 1) showed a segregation of three resistant to one sensitive in the F_2 generation, consistent with resistance in each case being due to the action of a single dominant nuclear gene. When R3202 and R3004 were reciprocally crossed, in the F_2 generation a ratio of 15 resistant plants to 1 sensitive plant was observed (Table 1), suggesting that the two genes *Lt1b* and *Lt2* are unlinked. Progeny of single F_1 plants of reciprocal crosses of heterozygous R2501-derived plants (*Lt1a/+*) with R3202 (*Lt1b/Lt1b*) were scored in the same way for resistance (Table 1). Three plants (C, D and E) gave no sensitive progeny in the F_2 generation, suggesting that they had received a resistance gene from each parent and that these two genes are allelic or closely linked. However, two plants (A and B) produced sensitive progeny, suggesting that they had received the wild-type sensitive gene from R2501; this hypothesis is supported for A by the segregation ratio of 33:8 (not significantly different from 3:1) and for B by the finding that, when 13 of the 15 resistant F_2 plants were self-pollinated and their F_3 progeny tested, 9 of them gave rise to some sensitive plants (pooled data 108:44 not significantly different from 3:1). No segregation data are available for the cross R2501 × R3004.

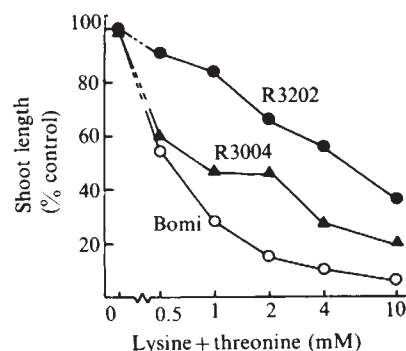


Fig. 1 Growth of two mutants R3004 (▲) and R3202 (●) and their parent cultivar Bomi (○) in a range of equimolar concentrations of lysine and threonine. Mature embryos were hand dissected and grown on a basal medium containing agar, salts, sucrose and vitamins in Petri dishes under lights at 25 °C as in ref. 5. Amino acids were filter sterilized before addition to autoclaved medium. After 7 days, 7–16 plants were removed and the length of the shoot from the tip of the first leaf to the base was measured. Results are expressed as percentages of the mean value of each genotype in the absence of lysine and threonine. 100% values ranged from 46 to 61 mm with standard error values less than 5 mm.

Table 1 Genetics of resistance to lysine plus threonine in three mutants crossed amongst themselves and with cv. Golden Promise

F ₁ parents female × male	F ₁ genotype at two loci		No. of F ₂ plants	
	<i>lt1</i>	<i>lt2</i>	resistant	sensitive
R2501 × Golden Promise	<i>Lt1a/+</i>	<i>+/+</i>	92	28
Golden Promise × R2501	<i>+/Lt1a</i>	<i>+/+</i>	50	18
R3004 × Golden Promise	<i>+/+</i>	<i>Lt2/+</i>	131	44
Golden Promise × R3004	<i>+/+</i>	<i>+/Lt2</i>	113	36
R3202 × Golden Promise	<i>Lt1b/+</i>	<i>+/+</i>	37	15
Golden Promise × R3202	<i>+/Lt1b</i>	<i>+/+</i>	74	27
R3004 × R3202	<i>+/Lt1b</i>	<i>Lt2/+</i>	183	13
R3202 × R3004	<i>Lt1b/+</i>	<i>+/Lt2</i>	151	10
R2501 × R3202 A	<i>+/Lt1b</i>	<i>+/+</i>	33	8
B	<i>+/Lt1b</i>	<i>+/+</i>	15	1
C	<i>Lt1a/Lt1b</i>	<i>+/+</i>	110	0
D	<i>Lt1a/Lt1b</i>	<i>+/+</i>	61	0
R3202 × R2501 E	<i>Lt1b/Lt1a</i>	<i>+/+</i>	58	0

Mature embryos were germinated for 1 or 2 days on basal medium before transfer to LT medium. Resistance was scored after 7 days' total growth. Resistant plants, when grown in the presence of lysine plus threonine, had long green shoots and pale scutella, whereas sensitive plants, derived either from the original selections or from control plants of several cultivars, had short yellow shoots and dark pigmentation on the scutellum and at the base of the leaf. The absence of darkening of the scutellum was used as an absolute indicator of resistance. Ratios were analysed by χ^2 test. In crosses with cv. Golden Promise the R2501-derived plant used was homozygous (*Lt1a/Lt1a*), in crosses with other mutants the R2501-derived plants were heterozygous (*Lt1a/+*). R3202 (*Lt1b/Lt1b*) and R3004 (*Lt2/Lt2*) plants were homozygous for resistance at the *Lt1* and *Lt2* loci, respectively. Crosses were performed with glasshouse grown plants. Flowers on plants of the female parents were emasculated and enclosed in cellophane bags for 2–4 days before pollination with anthers from selected male parents. Bags were kept over the ears until collected.

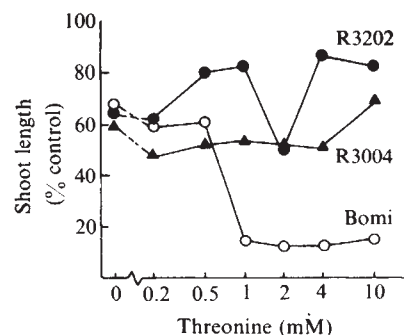
Aspartate kinase (AK) in barley can be separated into the three isoenzymatic forms AKI, AKII and AKIII (ref. 6); the latter two are sensitive to feedback inhibition by lysine. Analysis of the properties of these isoenzymes⁷ has shown that R2501 and R3202 both have an AKII with greatly decreased sensitivity to inhibition by lysine and a normal AKIII, whilst R3004 has an unchanged AKII but an AKIII that is relatively unaffected by lysine. These results, taken with the genetic data, suggest that resistance to lysine plus threonine in R2501 and R3202 is due to mutations in a gene *lt1* causing altered feedback sensitivity of AKII and in R3004 is due to a mutation in a gene *lt2* which decreases lysine inhibition of AKIII. The genes *lt1* and *lt2* are, therefore, likely to be structural loci for the two aspartate kinase isoenzymes. The absence of tight regulatory control of aspartate kinase would allow sufficient flux down the pathway to satisfy the methionine requirement in LT medium.

It has been suggested that mutants having such altered regulatory feedback properties might produce cereal plants with grains

Table 2 Soluble amino acids in grains of normal barley and three mutants

Amino acid	Soluble content (nmol per mg nitrogen)				
	Pooled controls I	Pooled controls II	R3004	R3202	R2501
Thr	9 ± 3	12 ± 5	116 ± 36	16 ± 5	147 ± 21
Lys	12 ± 5	12 ± 5	15 ± 3	18 ± 5	19 ± 8
Ala	49 ± 13	47 ± 10	41 ± 6	68 ± 14	29 ± 5
Val	18 ± 10	28 ± 12	17 ± 5	38 ± 10	19 ± 4
Glx	143 ± 25	140 ± 36	122 ± 20	199 ± 39	112 ± 22
Asx	167 ± 27	181 ± 28	122 ± 18	237 ± 45	131 ± 24
Total (17/20)	580	740	745	905	743
Seed nitrogen (mg N per g dry wt)	14–18	22–27	22–27	16–19	24–29

Milled grain samples were extracted with 5% cold trichloroacetic acid plus 10 mM 2-mercaptoethanol as in ref. 6 and analysed on a Technicon TSM autoanalyser. Values are expressed as mean ± s.d. for at least eight analyser runs and four grain samples. Tryptophan and cysteine were not determined; methionine peaks were always too small to quantify as they were overlapping with a small unidentified peak. Glutamine and asparagine were hydrolysed to the free acid before analysis. Data for R2501 are taken from ref. 6 for comparison and are for pooled seeds from heterozygous *Lt1a/+* individuals. Nitrogen was determined by Kjeldahl analysis. The two control groups were: I, Bomi and sensitive lines segregating from the original R3202 selected plant; II, sensitive lines segregating from the original R2501 selected plant.

**Fig. 2** Growth of two mutants R3004 (▲) and R3202 (●) and Bomi (○) in a range of threonine concentrations with lysine held constant at 3 mM. Mature embryos were grown and 12–19 plants measured as in Fig. 1. 100% values ranged from 49 to 67 mm with standard error values less than 5 mm.

of improved nutritional quality^{8,9}. Analyses of the soluble amino acids of seeds (Table 2) showed that the mutation in R3004 caused a greater than 12-fold increase in threonine. Similar increases have been previously observed in R2501 (ref. 6). The absence of such accumulation in R3202 may be because there was less of the mutant enzyme form⁷. An increase in threonine of 100 μ mol per g nitrogen would be sufficient to increase the total threonine content of the seed ($\sim$ 1,400 μ mol per g nitrogen) by about 7%. Increases of this order have been measured. This would decrease the amount of extra threonine required for optimum nutrition of monogastric animals fed on barley meal plus vitamins¹⁰. Seed of R3004 has been multiplied and will be used to test this in rat feeding trials. The agronomic performance of the R3004 line, even in the absence of outcrossing and reselection, is not markedly affected when compared with the parent Bomi.

None of the mutants has enhanced levels of soluble lysine. This is consistent with the lack of resistance to the lysine analogue aminoethylcysteine and is probably because the activity of the first enzyme unique to lysine biosynthesis—dihydrodipicolinic acid synthase—is also tightly regulated by lysine¹¹.

Variant plant cells accumulating tryptophan¹² and threonine¹³ as a consequence of altered enzymatic regulation have been selected. However, it is only possible to examine the effects of such changes on seed composition by using fertile plants. Our results confirm the view that it is possible to select mutants with relaxed feedback control which can accumulate nutritionally essential amino acids in the seed. A maize mutant which accumulates threonine in the seed¹⁴ has also been identified. We view these mutants as a significant step towards the development of a barley grain of balanced amino acid content.

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MATTERS ARISING

Zenith atmospheric attenuation measurements

ZAMMIT and Ade¹ have made a valuable contribution to observations of millimetre wave attenuation and rightly argue its importance in locating telescopes. In finding a linear relationship between attenuation and measured total water, they question the results of Llewellyn-Jones *et al.*² who reported from laboratory measurements a quadratic dependence of absorption on the amount of water. We believe that the two results may not be in conflict when the difference in degree of saturation of the vapour in the two experiments is recognized. In the laboratory the vapour sample was homogeneous in temperature and was measured over a wide range of relative humidity including values close to saturation. In the zenith sky experiment, the measurements represented integrated absorption from layers at different temperatures and of unknown relative humidity, the only other quantity known being the total amount of water. It was therefore possible for all layers to be sufficiently far from saturation so that the millimetre wave absorption was in the range of linear dependence on water amount. That this is realistic has been verified by modelling millimetre wave absorption for various atmospheric columns specified by radiosonde data taken as a function of height. These show that integrated absorption in real atmospheric columns can be an insensitive measurement from which to deduce molecular behaviour. We believe, therefore, that a monomeric water model is not proven to be adequate for the prediction of millimetre wave attenuation.

Zammit and Ade find that their measured absorption values exceed the

predictions of accepted standard models and while the validity of these must always be questioned, the procedure of simply reassigning parameters to fit a given set of experiments may obscure new effects. Any observed absorption in excess of a physically acceptable molecular model may mean that some additional mechanism has been overlooked. From the aspect of choosing telescope sites it then becomes important to see if meteorological conditions can be found when the additional mechanism is inoperative and the excess becomes zero.

Specifically, from what is known about excess absorption, there is a strong case for investigating continental sites surrounded by deserts such as Kitt Peak, Arizona or Sacramento Peak, New Mexico for comparison with maritime sites like Izana, Tenerife and Mauna Kea, Hawaii. It is possible that in dry regions the relative humidity values at all levels of the atmosphere would be lower and that telescope sites located in them would be free from the effects of local high humidity sources which were found at Mauna Kea³. Such a benefit would not be revealed by simply measuring the total amount of water above the site.

H. A. GEBBIE

*Department of Electrical Engineering
Imperial College of Science
and Technology,
London SW7 2BT, UK*

D. T. LLEWELLYN-JONES

R. J. KNIGHT

*Rutherford-Appleton Laboratory,
Chilton, Didcot,
Oxon OX11 0QX, UK*

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Petralona Cave dating controversy

IN their investigation of the fossil hominid cranium from Petralona Cave, Greece, Henning *et al.*¹ have neglected some of the relevant literature data. They state that the skull was lying on the cave floor. However, I² and all six men who found the skull³ report that the skull "was adhering to a rock"⁴ through a stalagmitic column, while the skeleton of the same individual was buried under a stalagmitic cover ~5 cm thick. The skeleton had apparently been separated from the skull during a dry period; the soil, having shrunk, moved the skeleton, while the skull remained attached by stalagmites about 20-24 cm above the skeleton. The skull belonged to a human who died there

before the formation of the travertine layer. Samples from the stalagmitic cover of the rock to which the skull adhered and from the travertine layer covering the skeleton, have been ESR-dated by Ikeya⁵ who obtained an age of 670 kyr BP (where the archaeological dose was 0.1 rad yr⁻¹) and 340 kyr BP (where the dose was 0.2 rad yr⁻¹). The same stalagmite tested by U/Th (sample 75 GR4-2) by Schwarcz *et al.*⁶ "actually yields a finite age of 440 kyr".

Hennig *et al.* state that the skull was covered only with a brown calcite layer. However, I maintain that the skull was covered with pale whitish stalagmite, similar to that which is still found on the rock of the 'Mausoleum' and which was adhering to the skull. The brown calcite was overlying the whitish encrustation of the skull⁷, which protruded by 7-10 cm.

The stratigraphy of the cave indicates an age for the skull and the skeleton of the Petralonian man older than 700 kyr. This is supported also by faunal evidence, macro and micro, preserved in the cave⁸⁻¹⁰ and by the lithic technique^{11,12} proving that man did live in the cave as early as the Lower Pleistocene.

ARIS N. POULIANOS

*Anthropoligike Hetaireia Hellados,
Daphnomele 5
Athens 706, Greece*

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IN their recent investigation of the age of the Petralona skull using electron spin resonance (ESR), Hennig *et al.*¹ compared the results of ²³⁴U/²³⁰Th dating², of the top travertine floor in the area where the skull rested. It is hoped that the contribution of the ²³⁴U/²³⁰Th dates (corrected for its detrital residues) to ESR results will clarify matters and be a step forward in resolving this controversy.

Their statement that practically all previous age determinations were done on samples from deeper strata, but not from the thin brown top layers of the travertine floor, was inaccurate. I have studied the top, brownish layer of the Mausoleum by the ²³⁴U/²³⁰Th dating method. Three samples in this investigation contribute to the ESR dates^{2,3}: P-12, P-13 and P-6 (Table 1).

P-12 had an obvious multi-layered appearance (consisting of an upper series of lime-reddish grey layers), ~0.6 cm thick which presumably implies different times of deposition. (A similar description applies to a further five samples throughout the cave.)

The age of P-12 drops from 250 kyr to 154⁺¹⁹₋₁₆ kyr using a mode of correction which calculates the different U and Th leachable radioisotopes, by analysing the detritus insoluble residues after total carbonic dissolution^{2,4}. This corrected age, due to the interstratified nature of the sample, indicating as it does a very slow deposition of calcite, (thin section analysis has confirmed this), represents the average of the intermittent stages of this 0.6 cm layer. That is, the upper surface layer could be as young as 80 kyr and the deeper part as old as 259 kyr, which would

Table 1 Radiometric data of uncorrected and corrected $^{230}\text{Th}/^{234}\text{U}$ ages of Petralona Cave travertines

Sample no.	Description	$^{230}\text{Th}/^{234}\text{U}$	$^{234}\text{U}/^{238}\text{U}$	$^{230}\text{Th}/^{232}\text{Th}$	U (p.p.m.)	Th (p.p.m.)	Age (kyr)*
P-12	Travertine (smooth and pale reddish brown), from upper part of Mausoleum calcitic floor	0.953 ± 0.045	1.272 ± 0.049	3	0.75	0.91	250^{+49}_{-34}
(U, Th) corr*		0.780 ± 0.042	1.171 ± 0.053	∞	0.59		$154.4^{+19.1}_{-16.4}$
P-13	Thick (1-cm) monocrystalline travertine from the upper part of Mausoleum calcitic floor	0.980 ± 0.046	1.315 ± 0.031	15	2.7	0.73	269^{+58}_{-39}
(U, Th) corr		0.781 ± 0.037	1.297 ± 0.032	∞	2.51		$150.2^{+15.2}_{-13.5}$
P-6		0.540	1.005	8	8.12†	1.70	84^{+46}_{-32} (130 kyr, +1 σ)

* (U, Th) corr, mode of correction and corrected ages.

† Severe bone contamination as determined by X-ray diffraction renders an age of at least 150 kyr + 1 σ the most probable. Small clay residue plus contaminant bone prevented (U, Th) corrections.

suggest an average growth rate of $\sim 0.003 \text{ cm kyr}^{-1}$.

The 4-cm travertine beneath (thick, unlaminate sheet of prismatic calcite), ranged in age from at least 750 kyr to 280 kyr (ref. 5).

P-13 gave an uncorrected age of 269^{+58}_{-39} kyr, which, when corrected for its detrital residue isotope ratios, became $\sim 150 \pm 40$ kyr.

Another indicative sample (P-6) presumably from the burial site of the skull, gave an age of 130 kyr (including 1 σ ; bone contamination noted). The appearance of this sample has many features in common with the material which encrusted the skull. The above age sequence was found to be typical for certain other parts of the cave.

However, any material, for example of prismatic calcite (colourless to pale brownish-grey), still remaining on the skull beneath the red-brown ESR-dated layer, would imply further that the skull is even older than 250 kyr.

From the above, we can see an obvious correlation, within the errors, between the U/Th series dates for the top travertine layer and the ESR dates for the stalagmitic encrustation on the Petralona cranium.

Y. LIRITZIS

Physics Laboratory II,
Patras University,
Patras, Greece

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THE determination of the age of the hominid cranium from Petralona by Hennig *et al.*¹ is remarkable because it is the first to date the carbonate encrust and cranium itself by the ESR method². However, there are uncertainties in their age derivation.

They assumed that the brown calcite encrusted the cranium soon after the death of the hominid. If this carbonate had been growing linearly up to recent time, the age of the cranium must be doubled to 400 kyr using their ESR age

for the carbonate. It must be stressed that the carbonate encrust age is just a minimum age of the cranium.

The high external dose rate of $190 \pm 20 \text{ mrad yr}^{-1}$ on the cave travertine floor is too high and is very close to that in the soil at Petralona. The dose rate in a limestone cave is generally highly affected by the flow-in soils nearby³. The high dose rate would be due to the soil mixed up by excavations in 1960–62 or recently.

The response of CaSO_4 to low energy γ rays is also high, leading to an apparently high dose rate. Furthermore, the carbonate encrust was presumably about a few centimetres above the cave floor and shielded by the cranium against the floor. Thus, it would not be appropriate to use the dose rate of 190 mrad yr^{-1} . If the external dose rate of 87 mrad yr^{-1} with thermoluminescent dosing of $\text{CaSO}_4(\text{Tm})$ or the $50\text{--}80 \text{ mrad yr}^{-1}$ of Liritzis *et al.*⁴ with $\text{NaI}(\text{Tl})$ at Petralona cave were used as the external dose rate, the total dose rate would be $\sim 100 \text{ mrad yr}^{-1}$. The average age of the carbonate encrust could then be 420–600 kyr which is close to those obtained for some carbonates and bones at Petralona with ESR⁵. Careful assessment of the radiation environment must be made using ESR dating before a large scale excavation proceeds.

We have used ESR to study Choukoutien bones. The total dose of natural radiation (TD) of Choukoutien bones estimated with ESR ranged from 20 to 40 krad. The TDs for Petralona bones range from ~ 10 krad for bones embedded in pure calcite to 80 krad in the soil sediment. The latter age would be ~ 400 kyr considering the high dose rate of 200 mrad yr^{-1} . The TDs of the cranium of 27 krad yr^{-1} obtained by Hennig is very close to that of bones of layer 17 at Petralona (21 krad) where Petralona man was originally located, according to Poulanos. There are some problems of apatite recrystallization in ESR dating of bones⁶. We might obtain a younger age than the real age for bones with ESR. However, these results may have some bearing on the stratigraphy. The bones at Arago, Tautavel fall in nearly the same

range of TDs.

Note added in proof: ESR dating of fossil bones are being determined taking ^{238}U -series disequilibrium and a model of constant uranium accumulation rate into account. Tentative ages neglecting the loss of gaseous radon are 810 kyr and 550 kyr (Kabuh and Pucangan Layer, Jawa), 335–480 kyr (Mauer, Heidelberg), 330–440 kyr (Petralona) and 240–200 kyr (Steinheim). Actual ages are older when a 30–50% loss of gaseous radon is taken into account.

MOTOJI IKEYA

Technical College,
Yamaguchi University,
Ube 755, Japan

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HENNIG *ET AL.* *REPLY*—With regard to the above comments we will keep our replies to each author distinct.

The statement by Poulanos in his own publications in *Anthropos* and *Current Anthropology* that there exists a skeleton which belongs to the same individual as the hominid cranium, that is said to have been “buried under a stalagmitic cover $\sim 5 \text{ cm thick}$ ”, clearly contradicts what he has published elsewhere²; “Unfortunately, the skeleton was not preserved and lost forever for science. Only the skull was preserved due to the fact that it was covered with stalactitic material”.

As far as we know, there is no indication that any bone fragments under the 5-cm thick stalagmitic floor are related to hominids, and even less to the hominid skull. Until now no proof for the existence of a hominid skeleton has been presented.

Poulanos furthermore quotes age data of 340 kyr and 670 yr derived by Ikeya (optionally for dose rates of 0.2 rad yr^{-1} and 0.1 rad yr^{-1} respectively³). Curiously, there is no detailed description of the colour, texture, thickness and exact sampling location of these samples in Ikeya's paper. Thus, it is almost impossible to

reconstruct a reliable stratigraphical relationship of these samples to the hominid skull. It is important to know that the skull was removed from Petralona Cave many years before Poulianos became involved.

With respect to the age cited by Poulianos of 440 kyr, which had been derived by Schwarcz *et al.*⁴, everybody can verify that this sample originates from a depth of >1.5 cm below the surface of the travertine floor at the discovery site of the cranium. Altogether four different samples of this clear, massive base layer have been dated by Schwarcz *et al.* (ref. 4, Table 1, samples 75 GR-4) and yielded the U-series ages 272.9^{+122}_{-68} , 451^{+150}_{-150} , 283^{+84}_{-57} and >450 kyr.

Based on these data, Schwarcz *et al.* have derived a growth interval for this clear base layer "from at least 450 to 260 kyr BP" (ref. 4, p. 161). Consequently, the overlaying (1.5-cm thick) calcite should be younger than 260 kyr, which is particularly true for the thin brown surface layer covering the hominid skull also. It must be stressed that Schwarcz *et al.* have only dated samples from the clear prismatic layer, but none from above this massive stratum (ref. 4, p. 155). Therefore, the age data of Schwarcz *et al.* cited above can only set upper limits for the age of the hominid cranium and its thin brown encrustation. (This is also true for the quoted ages derived by Ikeya³, provided he used the same stalagmite sample, as Poulianos maintains.) In this context, the lower ages of 273 and 283 kyr, respectively, and 260 kyr deduced by Schwarcz *et al.*⁴ are in agreement with our ESR age of 200 ± 40 kyr for the hominid skull and its covering brown calcite¹.

Regarding Poulianos' next point, one of us (N.I.X.) was involved in the preparation of the skull and removed the calcite encrustation that was adhering to the bone surface. Fortunately, on a few of these brown calcite pieces tiny bone fragments were attached, which we separated and used for an ESR-age determination¹. Poulianos obviously does not know that a part of the cranium base was intentionally left unprepared and is still today encrusted with the thin brown calcite layer. Here one can clearly see that this brown encrustation is directly in contact with the lamina externa of the skull bone.

As to his final point, we do not think that a stratigraphy alone can indicate any absolute ages, particularly as the hominid cranium is a surface finding. Any interrelation of the hominid cranium and the cave fauna is, therefore, very questionable. Moreover, the fauna at Petralona cave has also been described as Riss/Würm⁵.

Dealing with the comment by Liritzis, we are pleased to know that he also has studied the thin brown top layer at the site of the discovery of the hominid skull. He has recently given, for the first time,

details of three samples, P-12, P-13 and P-6, which were formerly only associated with the word 'Mausoleum' in his quoted publication⁶, in which, however, any further sample description is missing. Consequently, it was impossible to interrelate these three speleothem samples and thus they were not mentioned in our paper¹. We are, however, now pleased to recognize that samples of the thin brown top layer of the travertine floor were independently dated by the uranium series method. Certainly it is most remarkable that all three samples dated by Liritzis yield $^{230}\text{Th}/^{234}\text{U}$ ages in best agreement with our ESR ages of the hominid skull and its encrustation, even within the limits of error.

With respect to Ikeya, he doubts the temporal relation between the brown calcite encrustation and the skull itself and he further suggests we should double our age of 200 kyr by assuming linear growth until today. Surely it should be noted that here he does not rely on his own experimental data.

There are at least four arguments which point to a relatively rapid conservation of the skull by the brown calcite encrustation:

- (1) The excellent conservation of the osteological material of the cranium was recently confirmed by histological studies⁷ demonstrating that the skull was covered a relatively short time after the death of the hominid.
- (2) The ESR-dating of the skull bone material itself has yielded about the same age as its covering calcite layer¹.
- (3) A stratigraphically older calcite (sample(d)) which does not appear on the skull was taken 3–4 mm below the thin brown surface layer. Being older than the skull itself, it allows us to set an upper limit of 198 ± 50 kyr for the age of the hominid¹.
- (4) Ikeya's suggestion of a linear growth for the 1–2 mm-thick encrustation from 400 kyr until the present would imply a highly unrealistic growth rate of 1–2 mm per 400 kyr ($\approx 2.5\text{--}5 \times 10^{-3} \mu\text{m yr}^{-1}$) which is about three orders of magnitude lower than typical experimentally determined growth rates of 1–10 $\mu\text{m yr}^{-1}$ (ref. 8). Here, it may also be of interest that Ikeya published vertical growth rates of 0.3–0.6 $\mu\text{m yr}^{-1}$ and 0.45–0.9 $\mu\text{m yr}^{-1}$ for two different floor travertines of Petralona Cave³. In addition, the lack of lamination in the brown calcite encrustation indicates a relatively rapid and uninterrupted depositional event.

Another point is Ikeya's opinion that "the high external dose rate of 190 ± 20 mrad yr^{-1} on the cave travertine floor is too high". This contradicts his previous assertion³. "We are using the tentative annual dose of natural radiation of ~ 0.2 rad yr^{-1} for the Petralona stalagmite from U/Th measurements using low background Ge(Li)-detector"³.

With respect to our measured radiation

dose of 190 ± 20 mrad yr^{-1} , Ikeya recommends an external dose rate of 87 mrad yr^{-1} and a total dose rate of 100 mrad yr^{-1} . On the basis of this dose rate one would obtain ages between 270 and 420 kyr from our EDs of 27–42 krad (ref. 1) and not—as he claims—ages of 420–600 kyr. Obviously, there is an error in his simple estimation.

Furthermore, Ikeya does not say that he has measured his recommended dose rate of 87 mrad yr^{-1} —as far as we know—at some location in another branch of the cave, but not in the place where the hominid skull was found. Our dose rate of 190 ± 20 mrad yr^{-1} was established directly on the surface of the travertine floor at the site of the discovery.

In fact, relatively high dose rates on the surface of some cave floors are not unusual and similar values have been observed at other sites. For example, Ikeya reports a much higher archaeological dose for the surface layer of a Petralona cave floor as compared with deeper strata. His comment on this effect is simply: "Too much exposure at the surface?"³.

This effect of a comparably higher dose rate on cave floors was explained mainly by airborne radionuclides (for example radon and daughter products¹). Accumulation of atmospheric radon in calcite caves has been reported, and radon activities up to 13.2 mCi m^{-3} were for example, measured at Akiyoshi cave, Japan¹⁰. Such high radon concentrations will enhance the external α -, β - and γ -dose rates of surface layers in caves.

Furthermore, Ikeya reports a total dose (TD) that is an archaeological dose of 21 krad for "bones at the layer 17 at Petralona (21 krad) where Petralona man originally was according to Poulianos".

It is not surprising that using this measured TD value of 21 krad (determined by Ikeya) in connection with his suggested dose of 0.1 rad yr^{-1} yields an ESR-age for the Petralona hominid of 210 kyr.

On closer inspection, Liritzis' and Ikeya's data turn out to be consistent with our ESR-age determination of $\sim 200 \pm 40$ kyr for the Petralona skull.

G. J. HENNIG
W. HERR
E. WEBER
N. I. XIROTIRIS

*Institut für Kernchemie
der Universität Köln,
Zülpicher Strasse 47, 5000 Köln-1, FRG*

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BOOK REVIEWS

Science and technology, A to Z

Alan Isaacs

UNLIKE their editors, the reviewers of large reference books are not expected to read every word of the works they are asked to discuss. In the case of the new (fifth) edition of the *McGraw-Hill Encyclopedia of Science and Technology* one would have a full-time job for 2.4 years reading its 8.9 million words (assuming a 35-hour week and a reading rate of 2,000 words per hour). These figures highlight the enormous task of the editors, who have had to commission, edit, illustrate and index its 7,700 articles, which occupy some 12,700 A-4 pages arranged in 15 volumes. There were, so the blurb tells us, 3,500 contributors, 18 of whom were Nobel-prize winners. The result of this massive endeavour could have been a fiasco. In fact it is a *tour de force* of which its publishers have every right to be proud. It is extremely well illustrated and has been typeset and printed to a very high standard indeed.

Accepting the professionalism and authority of the work the first question that springs to mind — a £500 question — is “Who is it for?”. McGraw-Hill’s publicity suggests that libraries, teachers, students and general readers, not to mention scientists, engineers, technical writers, and industrial and business managers, will find it indispensable. This is a fairly high proportion of the population. But, as everyone knows, advertising copy cannot be taken seriously. Who *really* will find it useful?

Before attempting to answer this question one has to ask two others. At what level is it written and what does it contain? The editor, Sybil P. Parker, goes some way to answering the first of these questions in her preface:

The level of writing is, of course, consistent with the degree of complexity inherent in each subject, but is geared to nonspecialists.

The whole question of the level and style of writing is of the greatest importance in assembling works such as this. In this century the explosive growth of science in general, and the technology of communication in particular, has been accompanied by a blunting of the primary tool of communication — language. Technical language requires a technical vocabulary; scientists use a large number of words and expressions that are not found in the general language. Non-scientists find this puzzling and unacceptable. All too often they expect an intelligible explanation of,

The McGraw-Hill Encyclopedia of Science and Technology, 5th Edn. Edited by Sybil P. Parker. 15 volumes, pp.12,700. ISBN 0-07-079280-1. (McGraw-Hill: 1982.) £495, \$850.

say, quantum theory, in the vocabulary of the general language.

Another aspect of the same problem is syntactical. A writer needs a grasp of syntax that will enable his readers to understand his sentences after reading them only once. A scientist who does not have an inherent ability to say precisely what he means often loses confidence in himself and plunges into an ugly jargon, which he makes uglier still with impenetrable syntax.

Ms Parker and her team have managed to cope with both problems more than adequately. I am quite certain that not all the contributors presented typescripts containing the impeccable prose that has reached print. This is not to say that the layman will find many of the entries useful, or even intelligible. Ms Parker’s warning that the level is “consistent with the degree of complexity inherent in each subject” is appropriate and honest. No attempt has been made to simplify to an extent that involves either distortion or trivialization. For example, the section on special relativity uses such expressions as galilean transformation, inertial frame, ether drift, Lorentz–Fitzgerald contraction and Michelson–Morley experiment with either no explanation at all or a brief gloss. The article therefore does not pretend to inform the layman. Rather it is a summary that could be very useful to a physical scientist whose main field of study does not include relativistic mechanics. I am not sure what engineers would make of it; probably not much. This does not mean that the set is not a good investment for engineers. The civil engineer, for example, will find plenty of information in, say, mechanical, electrical and chemical engineering that he will undoubtedly find valuable. In general, then, the articles are designed for scientists and engineers trained in one discipline who are seeking information in another.

“Scientists”, in this context, should be taken to mean “hard” scientists, for the “soft” sciences do not find much place here. The publishers say that the work encompasses “all the physical, life, and earth sciences, as well as engineering. . .”. It is, therefore, something of a surprise to find

that one of the 75 headings in the useful subject index is *physiological and experimental psychology*. However, the remaining 74 sections together provide a detailed coverage of all of the hard sciences and of technology. The entries themselves are arranged in one alphabetical sequence opening with the inevitable and omnipresent *aardvark* at the beginning of Vol. 1 and closing with the obscure group of fungi *Zythiaceae* at the end of Vol.14. Between these alphabetical extremes the reader can learn about such a diversity of subjects as antisubmarine warfare, emotion, laser welding, open-pit mining, quantum chemistry, petrochemicals and the Wentzel–Kramers–Brillouin method (which may sound like an invention of Saki but is, in fact, a technique for solving the Schrödinger equation). Volume 15 contains a list of contributors, the topical index and the analytical index.

So, who should be spending £500 on the set. Clearly, at this price one is talking to librarians rather than individuals. One can say at once that for all libraries of science, technology and engineering it is an obvious must. But what about general libraries? How much of interest to the general reader does it contain that is not available in the latest edition of, say, *Encyclopaedia Britannica*? This is a critical question for individual librarians, and one to which there is no definitive answer. While *Encyclopaedia Britannica* is strong and authoritative in the sciences, the *McGraw-Hill Encyclopedia* is much more detailed and almost a decade more up-to-date. One can only generalize: any library that has a serious intention of catering for scientists, technologists and engineers needs a copy of this edition of the McGraw-Hill opus.

Finally, a few words of criticism, in the hope that they may be helpful in preparing future editions. I was surprised to find no mention of the now widely current expressions *electric constant* and *magnetic constant*; the articles under permittivity and permeability are adequate except for the omission of these modern terms. In the article on the plant kingdom the proof-reading has not been as good as it should have been, and the somewhat controversial classification provided is, I am told, not acceptable to all plant scientists, but the article does not say so.

The alphabetical index can sometimes be very helpful and sometimes not. For

example, in view of the current controversy I tried to find what the *Encyclopedia* had to say about Darwinism. I was surprised to find that it was an entry neither in the main text nor in the index. There are, it is true, 11 references in the index under *Darwin, Charles*. None of these proved helpful, however, and the reference to the *Origin of Species* turned out to be no more than a brief mention in an article on plant taxonomy. After an inordinate amount of time I found what I was looking for in the article on speciation. But, incidentally, the set is silent on cladistics — at least I think it is, because it does not appear in the index. □

Alan Isaacs is editor of the Macmillan Encyclopedia, the Penguin Dictionary of Science, Longman's New Dictionary of Physics, and science editor of Collins English Dictionary. He has contributed to many other dictionaries and encyclopaedias.

Tests of gravity

Joseph H. Taylor

Theory and Experiment in Gravitational Physics. By Clifford M. Will. ISBN 0-521-23237-6. (Cambridge University Press: 1982.) £37.50, \$75.

GRAVITATION, the only one of nature's basic forces that we ordinarily feel, has been the centre of renewed research effort in the past quarter of a century — largely because improved experimental techniques have made possible ever more precise tests of theory. In a concise and meaty book, Clifford Will has summarized the recent developments in the field, and has brought together a store of information not otherwise available in one place. It is a truism that the fate of a physical theory lies in its successful prediction of the results of experiments. With this fact clearly in mind, Professor Will presents his material by concentrating on the methods and results of both laboratory and astronomical tests of the subtleties of gravity, and on the theoretical tools used for interpreting the experiments.

The first quarter of the book presents the theoretical foundations of the subject, from the Einstein Equivalence Principle (in a sufficiently small, freely falling enclosure, it is impossible to tell whether gravitational forces are present) to the detailed mathematical framework of gravitation as geometry. We then encounter the Parametrized post-Newtonian (PPN) formalism, a theory of gravitational theories developed by Kenneth Nordvedt towards the end of the 1960s and later extended in collaboration with Professor Will. The PPN framework is well suited to the analysis of the slow-motion, weak-field experiments that can be performed in the

laboratory or with Solar System astronomical objects; in such a post-Newtonian limit, any metric theory of gravity can be characterized by the values of a set of ten parameters, the so-called PPN parameters. For general relativity, two of the parameters have value unity and the rest are zero; in the post-Newtonian limits of other theories, the parameters have other values. The book presents detailed recipes and examples of how the parameters can be calculated within a given theory.

The middle section of the book develops the equation of motion for bodies moving under post-Newtonian conditions, and then uses this formalism to analyse the "classical" tests of general relativity: the deflection of starlight near the limb of the Sun, the time-delay of radar signals in the vicinity of the Sun and the perihelion shift of the orbit of Mercury. Together with the results of some less famous experiments involving lunar laser ranging, geophysical effects, the orbits of planets and the constancy of the Newtonian G , these tests establish a set of experimental constraints on the PPN parameters. We learn that "viable theory space" is squeezed to the extent that in the post-Newtonian limit, any gravitation theory must agree with general relativity to within fractional margins of error ranging from 1 to 10^{-7} per cent.

Unfortunately, perhaps, there are a number of distinct theories of gravity which can, by suitable adjustments of arbitrary parameters, accommodate all of the Solar System experiments. Consequently, the last quarter of the book deals with tests which probe beyond the weak-field, post-Newtonian limits. These tests involve gravitational radiation, the structure and motion of neutron stars and other inherently relativistic compact objects, timing measurements of the orbiting pulsar discovered in 1974 and, finally, observations of cosmological significance. Only the binary pulsar experiment has yet produced results of quantitative significance; again, the results are consistent with general relativity, while constraining other theories rather severely.

Why so much ado about "other" possibly viable theories of gravity, when relativity remains uncontradicted by experiment and stands alone in its freedom from arbitrarily adjustable constants? True, the failure of all attempts to quantize relativity still segregates it from the rest of modern physics, but then the other theories being discussed are not quantum theories either. So the motivation for undertaking the hard labours involved in developing or mastering the PPN machinery seems obscure. Nevertheless, I find Professor Will's book a valuable addition to my library, and a most useful reference work. Other researchers and serious students of gravitation should be pleased with it, too.

J.H. Taylor is Eugene Higgins Professor of Physics at Princeton University.

Seen in colour

W. R. A. Muntz

Comparative Color Vision. By Gerald H. Jacobs. Pp.209. ISBN 0-12-378520-0. (Academic: 1982.) £16, \$24.

THE opening words of the preface to *Comparative Color Vision* describe how the author received a telephone call some years ago asking how to go about studying colour vision in the American elk. Anyone who has worked on comparative colour vision will have received similar requests, and will have shared Dr Jacobs's frustration that no convenient and up-to-date source of information is available on the methods and results of work in this area.

The present book is Dr Jacobs's response to this situation, and about half of it does indeed give the required information both clearly and concisely. Thus an initial chapter sets out the necessary techniques, dealing particularly with the bugbear problem of brightness, and should help anyone designing experiments to avoid the major pitfalls. Two further chapters late in the book present a comparative survey of colour vision in vertebrates (invertebrates are specifically excluded). These chapters are especially comprehensive in their coverage of the mammals, particularly the primates: 36 of the 58 pages of the survey deal with the mammals, and of these 20 are devoted to the primates. This emphasis is not a defect, for it is only reasonable for a book to reflect the author's interests, but it inevitably results in the "lower" vertebrates being more sketchily covered. With this minor proviso, these chapters provide a valuable introduction to the field.

The rest of the book is mostly devoted to background information on vision in general, with accounts of the structure of the eye, photopigments, basic electrophysiology, the psychophysics of human colour vision and so on. I feel these sections are less useful, since many other publications exist which deal with these subjects well. It is also impossible, in a book of this length, to cover such a range of topics in any depth, and it seems to me that in this part of the book the author has fallen between two stools: the information is so condensed that it would be difficult for anyone who is not already familiar with the field to understand it, and yet for those who are experienced it will add little to their knowledge or understanding.

In spite of these reservations the book serves a useful purpose. Anyone working within the general area it covers will find both interest and a number of new facts, and it should in particular provide a very useful introduction for someone who knows the basic data of vision, and now wishes to pursue the subject in colour. □

W.R.A. Muntz is Professor of Biology at the University of Stirling.

Everything you wanted to know about . . .

Leslie Crombie

The Natural Coumarins: Occurrence, Chemistry and Biochemistry. By Robert D.H. Murray, Jesus Mendez and Stewart A. Brown. Pp.702. ISBN 0-471-28057-7. (Wiley: 1982.) £70, \$160.

MORE than 800 natural coumarins are known today, many from higher plants, but a number are important compounds from microbial sources and include antibiotics as well as the more notorious group of aflatoxins. With the appearance of this large and well-printed volume there can be few groups of natural products which have so modern and extensive a documentation; indeed if there were more volumes of this type natural products study would become much more accessible, and some order brought to a massive and rather sprawling area of science.

The volume should attract more than one type of reader and in some ways it is two books in one. Chapters 1-8 will be of special interest to natural products organic chemists interested in the themes of structure, synthesis and biosynthesis. These chapters are particularly impressive for their depth of coverage, bringing together much scattered information. The style of writing is pleasant, and older information is integrated with newer material in a way which gives the reader confidence in the judgement and scholarship of the authors. There is much here in the chemistry, synthesis and use of spectroscopic and other techniques to attract the non-coumarin specialist.

Chapters 9-13, the second part of the text, have biochemically orientated themes which tend to concentrate on certain biologically active structures such as the aflatoxins, dicoumarol, novobiocin and the furanocoumarins. All these compounds have interesting biochemical interactions which lend themselves well to an essay-type of treatment. Some are of more recent discovery, but knowledge of the effects of the furanocoumarins (e.g. from rue) goes way back in time, and photosensitization reactions in skin by their linear members are now well studied. An indication of what our ancestors thought about the biological activity of certain plant furanocoumarins is given in the concise lines,

Rue maketh chast and eke preserveth sight,
Unfuseth wit, and Fleas doth put to flight.

These claims, however, are perhaps somewhat excessive.

Despite a careful exposition of the different numbering systems for coumarins given early in the book, it is unsettling that two systems are employed in different parts of the text for furanocoumarins, one intended for chemists, one for biochemists and biologists. There are instances of interesting but unreferenced items, such as chlorflavonin (p.101), but few errors jar

the reader's pathway. Nonetheless the strong recommendation, in heavy black type, of a total ban on benzene for extraction and chromatography on the grounds of toxicity hazards, does seem a little excessive. If professional chemists cannot handle toxic materials by safe working, who then will?

Impressive features of this book are the indexes and tables which must have involved a remarkable amount of work. These cover 379 pages of the 702-page book. Natural coumarins are tabulated according to chemical type along with trivial name, empirical formula, melting point, rotation and references. There are then separate indexes of melting point, molecular weight, trivial names, botanical occurrence, and families and genera, together with a general index and 3,383 references — we shall henceforth have little excuse for not being able to find specific pieces of information on natural coumarins. This is a massive work, with strong elements of original treatment, which will become a valued and well-known book among scientists in the natural product area. □

Leslie Crombie is Sir Jesse Boot Professor of Organic Chemistry at the University of Nottingham.

Reprise of calcium and secretion

O. H. Petersen

Calcium and Cellular Secretion. By Ronald P. Rubin. Pp.276. ISBN 0-306-40978-X. (Plenum: 1982.) \$35, £22.05.

THE importance of calcium in the control of secretion has not been in dispute for many years. However, the realization of the key role played by calmodulin, the demonstrations of the internal calcium control of membrane conductance, the characterization of calcium channels and the availability of much improved methods for measuring the internal free calcium concentration, together with many other important findings made in the past ten years, is certainly justification for a new book on the subject. This monograph is a much improved version of *Calcium and the Secretory Process* by the same author, which appeared in 1974 (for review see *Nature* 255, 265; 1975).

The present book is only slightly longer than its predecessor, but contains much new material reflecting the rapid growth of

this research field. There are four main chapters. The first deals with a mixture of general problems such as analytical techniques, subcellular calcium distribution and its redistribution during stimulation. There is a wealth of useful information including a critical assessment of calcium antagonists. Unfortunately the sections on calcium channels and the effects of internal calcium on other ion channels are already out of date, since there is no mention of single-channel current recordings with the Neher/Sakmann patch clamp technique. Dr Rubin can hardly be blamed for this, since the results of the application of this powerful technique to secretory cells have only recently been appearing in print. More worrying is the author's tendency to quote reviews rather than original papers. This can have unfortunate consequences as seen in the discussion of the calcium-evoked potassium release, where no reference to Gardos is found although this phenomenon is generally associated with his name.

The second chapter is a survey of calcium actions covering all the main secretory cell types. The historical development of the stimulus-secretion coupling concept is traced by considering first the cholinergic nerves and then the adrenal medulla and the neurohypophysis, but perhaps because these three systems are discussed mainly in a historical context one looks in vain for even a brief description of the Baker-Knight experiments with permeabilized adrenal medulla cells (*Nature* 276, 620-622; 1978). It is sad that an otherwise fine chapter, which is on the whole up to date, should be marred by the failure to discuss one of the most direct approaches to the question of intracellular control of secretion.

Chapter 3 is a brief consideration of possible mechanisms by which calcium acts to control secretion. There is rightly emphasis on calcium-binding proteins and in particular calmodulin, but it is clear that many important pieces of the puzzle are still missing. Finally, the author deals with the interactions of calcium with other putative intracellular mediators, in particular cyclic AMP and cyclic GMP. The picture is far from clear, but this again is not Dr Rubin's fault. These are muddy waters and much remains for research workers to discover and probably also "undiscover".

Dr Rubin's compact monograph will be useful to the many students of this exciting research field. It is not yet the ideal book on the topic, but considering the difficulties in compressing the vast amount of available material into what has become a very readable book, we must be grateful for this effort. □

O. H. Petersen is George Holt Professor of Physiology at the University of Liverpool, and author of *The Electrophysiology of Gland Cells* (Academic, 1980).

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